IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of

Anderson	Confirmation No. 7327
Serial No. 09/994,937	Group Art Unit 1616
Filed November 28, 2001	Examiner Abigail Fisher

For **SOLVENT SYSTEMS FOR PHARMACEUTICAL AGENTS**

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

DECLARATION OF DAVID M. ANDERSON UNDER 37 C.F.R. 1.132

David M. Anderson declares as follows:

1. I am the inventor of the above-identified application. I hold a position in Lyotropic Therapeutics, Inc., the assignee of record of the above-identified application, as Vice President, Scientific Affairs. I have read and understand the application, and I have read and understand the office action mailed March 16, 2009, and its cited references. I am also the inventor of the WO 99 12640 to Anderson (hereinafter "Anderson"), and Lyotropic Therapeutics is the owner of national stage filings made from Anderson.

2. I have previously submitted Declarations in this case, including those filed on October 14, 2008; March 26, 2009; and December 16, 2009. From these declarations it can be seen that I am qualified as an expert in the field where the present invention is made (chemical formulations and drug delivery, particularly including structured fluids (e.g., reversed cubic phase and other materials), and my experience, education, qualifications, and expertise remain accurate and true.

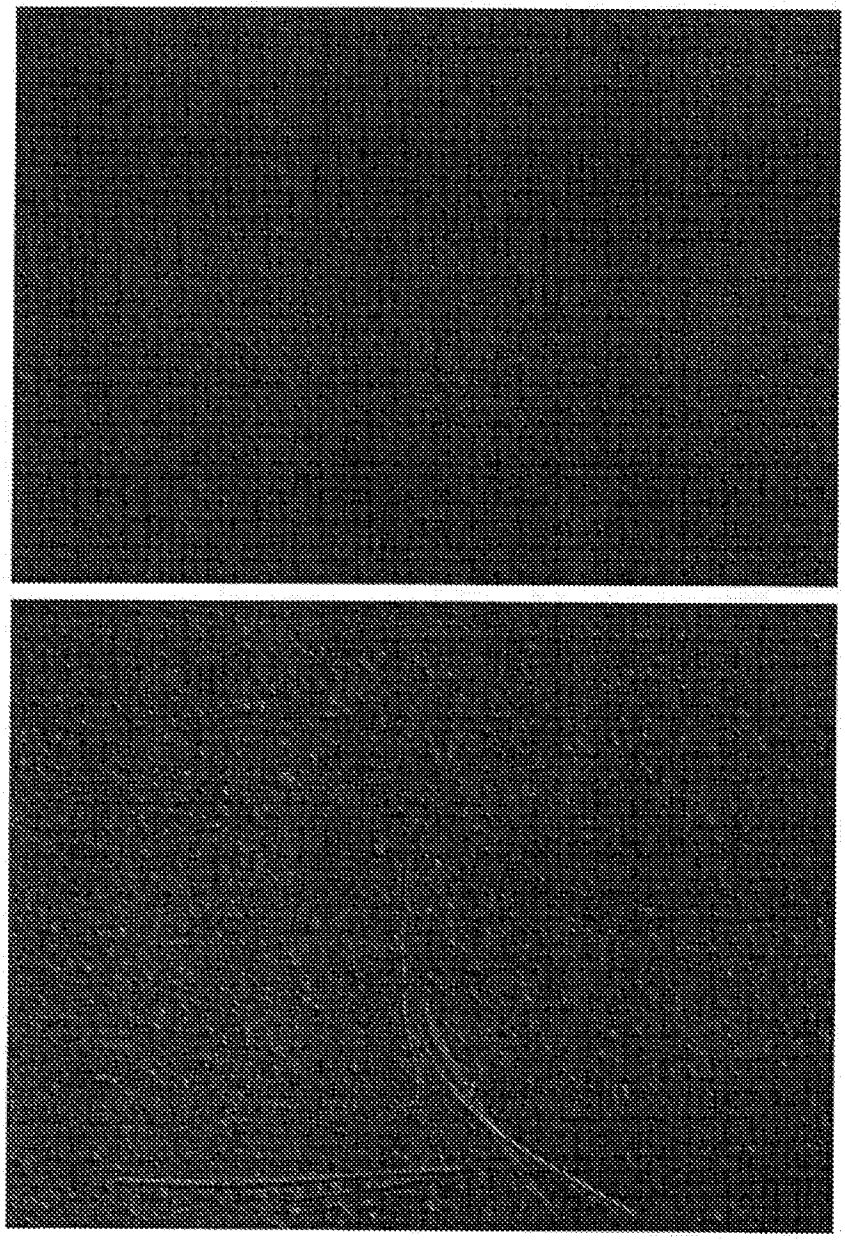
3. A particular focus of the instant invention is the solubilization—with true *thermodynamic stability*—of drug actives within somewhat rare and exotic reversed liquid crystalline phase materials that the inventor deems to be of high value in drug formulation and delivery. This was

not achieved in Examples 36 and 37 of Anderson, as is clearly stated in each Example. Example 36 states: "… precipitation of some of the Paclitaxel within the interior of each particle may occur since the solubilization of Paclitaxel … at this high loading was metastable." (emphasis added). Example 37 similarly states: "The concentration of Paclitaxel in this example was high enough that the solubilization was metastable, which has implications as discussed in the previous example."(emphasis added)

4. The contrast between Anderson and the present invention is demonstrated dramatically in the two photomicrographs below from my laboratory which point out an inherent and crucial distinction of the instant invention. The top figure shows an embodiment of the Anderson patent, in which a cubic phase containing the drug bupivacaine (free base) was prepared at a composition in which the bupivacaine was metastable, i.e., supersaturated, so that over time some amount of bupivacaine would precipitate forming bupivacaine crystals. However, due to the fact that the Anderson teaches crystalline-coated particles, any precipitation of bupivacaine over time due to the metastability would be confined inside the coated particles of that invention. Thus the complete solubility of the drug in the cubic phase material was not present and was not necessary to the formulation. In the photomicrograph of the dispersion 3 months after preparation, no bupivacaine crystals (nor crystals of any kind) micron are visible in the formulation. This precipitation confinement has been observed in the inventor's lab in a number of coated particle systems.

In contrast, the bottom figure shows the same cubic phase composition as in the coated particles of the top figure, only 1 month after preparation, as a dispersion of *uncoated* particles. Clearly, very large crystals of precipitated bupivacaine are visible, evidencing the metastable nature of the composition. Because coated cubic phase particles are but one of many possible formats for cubic phases in drug formulation, with certain advantages and disadvantages for certain routes of administration, means of sterilizability, drug release profiles, and pharmaceutical elegance, the inventor has developed other formats (e.g., U.S. 7,713,440) in which the cubic phase is not coated. Such formulations require the true solubilization of drug in cubic phase material, that is, a truly stable single thermodynamic phase which remains a single thermodynamic phase over time.

DIC optical micrographs of: (top) coated cubic phase particles after 3 months storage; and (bottom) uncoated cubic phase particles at the same cubic phase composition showing very large precipitated crystals of the drug, bupivacaine, after only 1 month.



5. It is well known to anyone skilled in the art that precipitated crystals larger than a few microns in size, such as those seen in the bottom micrograph, can be lethal upon injection, by

creating pulmonary emboli in particular. Indeed, it can be said that the career of the highly distinguished researcher Samuel Yalkowsky was largely devoted to this issue. See, e.g., Yalkowsky SH, Krzyzaniak JF, Ward GH. (1998) *J Pharm Sci.* 87(7):787-96. Therefore the difference between the metastable mixtures of the Anderson prior art and the stable solutions of the instant invention can literally be the difference between life and death. Other problems caused by precipitation of metastable mixtures include lack of content uniformity, requirements of difficult filtration procedures, loss of expensive actives, batch-to-batch inconsistencies, etc.

6. While the word "solubilizing" is used in Example 36, one of ordinary skill in the art would be well aware that Example 36 does not teach a single thermodynamic state being created (e.g., a true solubilization). A careful analysis of the wording used in Example 36 will have direct bearing on what would be obvious to one of ordinary skill in the art. Some of the distinctions are as follows:

A. Although the opening sentence of Example 36 states that "... was prepared by solubilizing...", it becomes clear as the Example continues that this mixture was not a true solubilization, but only a metastable mixture. While the expression "metastable solution" is an oxymoron and not technically correct, it is in fact frequently used, as it is short and descriptive. Similarly, while the "solubilization" in Example 36 was repeatedly stressed to be not a true, stable solubilization, what the preamble should be taken to mean is that a metastable 'solution' was prepared, and subsequently found to not be stable. Indeed, in the context of the instant invention, one would perceive Example 36 to be a *failure*, and the instant invention a resounding success in producing drug-loaded cubic phase compositions with consistently high enough drug loadings to be pharmaceutically useful, e.g., in injectable products.

B. Although Anderson refers to the mixture as a cubic phase, this is erroneous, and as stated twice in Example 36, the mixture is in fact a *liquid phase*, not a liquid crystalline phase. The use of glycerol (instead of water) in the initial mixture and a glycerol-rich solution in the exterior phase precludes the possibility of a cubic phase at this composition. The Anderson invention applies to both nanostructured liquid phases and liquid crystalline phases, and one of

the purposes of Example 36 is to describe an embodiment wherein the particle interior is a nanostructured liquid phase. In short, *Example 36 does not even contain a cubic phase.*

7. The present application defines "**Solubilize**" as "...a compound under consideration is solubilized in a liquid or liquid crystalline material if and only if the molecules of the compound are able to diffuse within the liquid or liquid crystalline material as individual molecules, and that such material with the compound in it make up a single thermodynamic phase." That this is in agreement with thermodynamic stability as known to one skilled in the art is demonstrated by, e.g., V. Vitagliano (1991) *Pure & Appl. Chem.* 63(10):1441 and U.S. 3,966,962 to Yalkowsky. (See also U.S. 6,348,215 to Straubinger). It is well known in the art that a requirement for true thermodynamic stability of a solution is that the solution be absent from precipitation upon prolonged storage (taking into account the possible confounder of chemical degradation, and taking steps to limit such). Methods and associated criteria for determining the solubility of a drug in a given solvent are well known in the art, and include prolonged storage as well as measurement techniques such as HPLC, etc. See, e.g., U.S. 3,966,962 to Yalkowsky, and specifically for the case of paclitaxel, P. Constantinides et al. (2000) *Pharm. Res.* 17(2):175.

8. In my opinion, for the reasons noted above, Anderson does not show or suggest a reversed cubic phase in the particle where a difficult to solubilize compound is solubilized in the reversed cubic phase such that a single thermodynamic phase is created.

9. While the Examiner cites Burdock and Muldoon to state that anisole should be considered an essential oil component, in my opinion one with ordinary skill in the art would not consider it to be such, for the following reasons.

A. As known in the art, and as inferred from each of these two references, anisole (e.g., as used in the Anderson reference) is obtained not from a plant source but rather by synthesis. Burdock teaches that, according to the Code of Federal Regulations 21 CFR 172.515 anisole is a *synthetic* flavoring substance. Muldoon exhibits the reaction of sodium phenoxide with methylsufate to produce anisole. Anisole as obtained from suppliers, such as Aldrich, is synthesized, not extracted from a plant.

B. A highly definitive reference, namely the Merck Index, in its "anisole" entry (#699 in the 12th edition) cites 4 synthesis routes for anisole, but nowhere in the 150+ word entry is any natural source mentioned. Likewise, the entry for "oil of anise" (#6691) nowhere lists anisole as a substantive component. Less definitive references sometimes cite anisole as a major component of anise oil only because they have confused anisole with anethole, the latter of which is indeed the major component of anise oil, 80-90% of the oil according to the #6691 Merck entry. Likewise, a PubMed search which I conducted in September 2010 using the keyword "anisole" results in over 400 scholarly articles, whereas crossing "anisole" with "anise" results in—*zero* hits.

C. While Burdock lists apple, cheeses, butter, olive, Bourbon vanilla, Jerusalem artichoke, sapodilla fruit, truffle and certain animal tissues that contain anisole, none of these are the source of any essential oil that would be known to someone with ordinary skill in the art. Furthermore, as in the case of anise oil, the anisole contents of these materials (were they to exist as essential oil extracts) would be very small.

D. U.S. 5,665,386 to Benet el al. lists an impressively thorough listing of essential oils and components thereof, which could well be taken to represent the state of knowledge of one skilled in the art. Nowhere is anisole listed—even though anise oil is listed.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David M. Anderson, Ph.D.

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MINIREVIEW

Formulation-Related Problems Associated with Intravenous Drug Delivery

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Introduction

The intravenous route is the most rapid and the most bioavailable method of getting a drug into the systemic circulation. However, this route is prone to a number of problems. Some of the problems associated with intravenous drug delivery are independent of the formulation. These include hypersensitivity, microbiological contamination, particulate matter, and poor injection technique or burred needles. These have been discussed in detail by Turco[1] and in books edited by Avis et al.[2,3] Others are the result of design of the formulation itself and the rate at which it is injected. The major adverse effects of intravenous administration that result from the formulation are hemolysis, precipitation, phlebitis, and pain. These latter adverse effects are the subjects of this review. Each is discussed with respect to its relationship to the design of the formulation and the way in which it is injected. A single model of the injection site is presented to aid in the understanding of the causes of hemolysis, phlebitis and pain, and their relationship to precipitation of the active ingredient.

Hemolysis is the loss of integrity of the red blood cell membrane with the release of the cellular contents into the plasma. The resulting increase in hemoglobin concentration can be particularly problematic. If the release of free hemoglobin into the circulation is more than the body can clear, a number of symptoms can result, including fever, chills, abdominal and back pain, shortness of breath, prostration, and shock. High plasma concentrations of hemoglobin can lead to plaque formation and the clogging of renal tubules, thereby affecting kidney function.[4-6] Hemolysis may also produce congestion in the reticuloendothelial cells of the spleen and liver causing splenomegaly and jaundice, respectively.[7] Hemolysis, or the lysis of red blood cells, can result from hypotonicity or from the effect of either the drug or the formulation components on cell membranes.

The only purely physical problem associated with the intravenous injection of drugs is phase separation, i.e., the formation of oil droplets or crystals of drug upon mixing of the formulation with blood. Precipitation of solubilized drugs can also result in uneven or delayed bioavailability. In fact, the mere presence of a separate drug phase indicates a reduced concentration in the aqueous phase. If the second phase is rapidly redissolved there may be no loss in total efficacy. However, if it becomes embedded into or sorbed onto the cells of the vein wall and redissolution is gradual, either reduced or prolonged efficacy can result. If the precipitated material is crystalline, the particles can cause cellular abrasion as they move along the vein wall.

Phlebitis (or thrombophlebitis) is an inflammation of the vein wall. It is similar to other inflammatory processes in that it is characterized by clinical observations of pain tenderness, edema, erythema, and a local temperature increase. In addition, phlebitis can cause thrombus formation which can ultimately lead to death. Phlebitis is a fairly common and potentially very serious side effect of intravenous therapy. Boon et al.[8] found that drugs commonly used in anesthetic practice produce a 36% incidence of phlebitis. Phlebitis has also been reported as a common side effect of amiodarone,[9] phenytoin,[10] and diazepam.[11] Phlebitis has long been associated with the mechanical and chemical effects of particulate matter introduced during injection. These particles may be present initially in the

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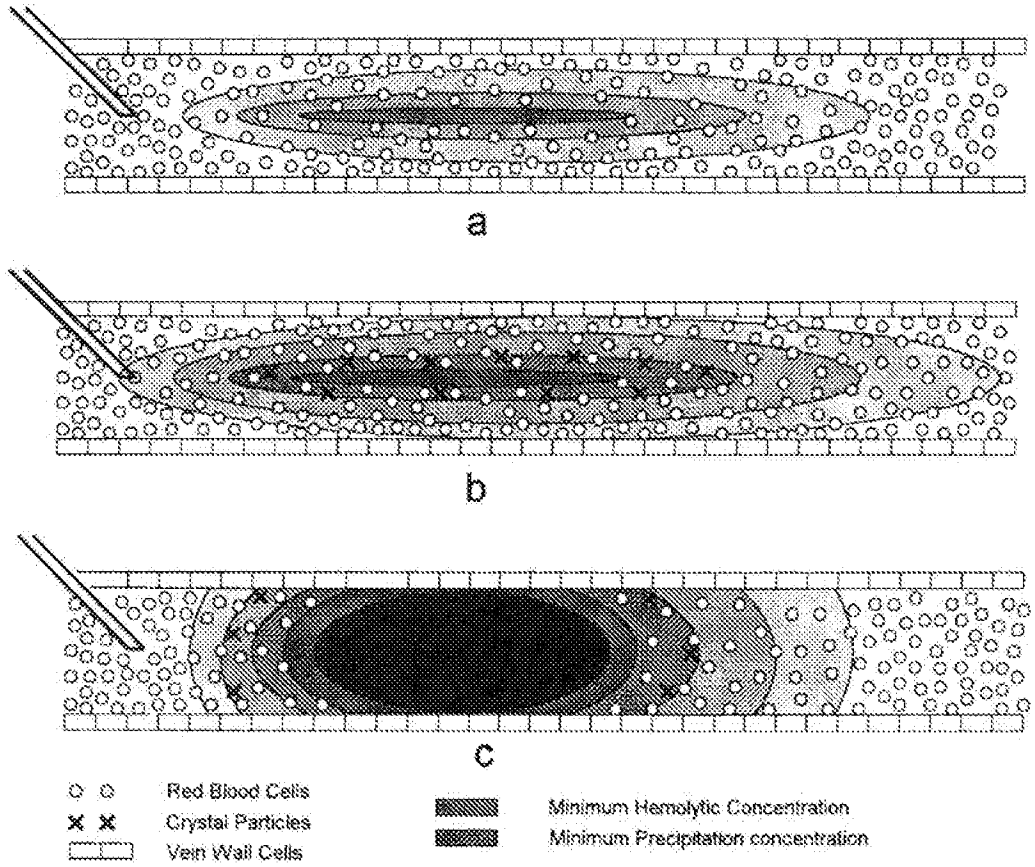

Figure 1—Schematic of intravenous injection site. (a) slow injection rate, (b) moderate injection rate, (c) very rapid injection rate.

formulation, due to a physical instability of the formulation during storage, inadequate quality control over manufacturing, or poor extemporaneous compounding technique. Additionally, spontaneous crystallization of the drug at the time of administration may occur if drug solubility limits are approached during dilution and mixing in the bloodstream.[12] Phlebitis can also be related to the inherent properties of the formulation components.

Both the cause of pain and its preclinical evaluation are poorly understood. In some cases, but not always, pain is associated with tissue damage and is reflected by either hemolysis or phlebitis. Graham et al.[13] showed that while 87% of patients receiving intravenous diazepam experienced pain, less than 10% manifested evidence of phlebitis.

This paper reviews the causes of the above problems and the various in vivo and in vitro means available for their evaluation. The results of studies conducted using both in vitro and in vivo methods for the detection and prediction of physical/chemical changes in the formulation during delivery are discussed. A general model of the injection site is presented in order to aid in understanding the physical and biological consequences of formulation interactions in vivo. Using this model the interrelationships among hemolysis, precipitation, phlebitis, and pain, with respect to the design of intravenous formulations, are discussed.

Injection Site Model

To minimize or totally eliminate problems associated with hemolysis, precipitation, phlebitis, or pain resulting from intravenous injection, it is necessary to have good (preferably in vitro) screening procedures for these problems. Before such procedures can be developed, it is helpful to have a reasonable model for the injection site. The model described in Figure 1 can be helpful in understanding the factors that contribute to both hemolysis and phlebitis.

As the formulation is injected it forms a plume that is carried downstream by the blood. The plume is most concentrated (darker) in the center and becomes progressively more dilute (lighter) with increasing distance from its center. As the formulation diffuses, the plume becomes larger and less concentrated with increasing distance from the injection site. Injection of the same formulation at a slower injection rate (or into a larger vein) will produce a lower concentration of formulation at all points because any given amount of the formulation will be diluted by a greater volume of blood after a slow injection. A moderate injection rate produces a wider and more concentrated plume. These are illustrated in Figure 1, parts a and b, respectively. As the plume moves downstream it is diluted and enlarged by blood from intersecting veins. Eventually it is diluted to a negligible concentration spread over the entire blood volume. If the formulation is injected very rapidly, i.e., at a rate which is greater than the blood flow rate, it will fill the vein and form a formulation plug as illustrated in Figure 1c instead of a plume. In all cases, once the injection is completed, the red blood cells move downstream at about the same rate as the formulation plume. Note that the actual flow rate depends on the size of the injected vein and the physical condition of the patient.

Hemolysis—Hemolysis will occur if the red blood cells are placed in an environment which is hypotonic or which can cause the disruption of their membranes. Although hemolysis is not strictly proportional to solution tonicity it is definitely a function of vehicle composition.[14–16] It can be caused by cosolvents, surfactants, and other excipients

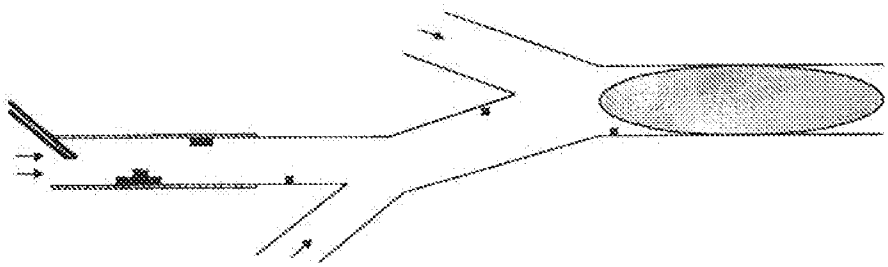

Figure 2—Schematic of injection site and downstream veins after injection (dark areas represent precipitated particles).

as well as by the drug itself. Nearly any hemolytic solution will become less hemolytic when diluted with normal saline or with blood.

Hemolysis is not an instantaneous process. It takes time for enough drug to interact with the cell membrane to alter its function or for sufficient water to enter the cell to cause it to rupture. Just as it is possible to pass a finger over a candle flame without getting burned, it is possible for a red blood cell to pass through a hemolytic condition without damage. In both cases time is the key factor. All cells can withstand an undesirable condition for a short time. The time required for a condition to produce cell damage is inversely proportional to the severity of the condition. Thus red blood cells can endure a slightly hemolytic concentration of a substance longer than they can a highly hemolytic concentration.

When a formulation is injected into a vein, the red blood cells (circles) are displaced from the center (darker region) by the formulation itself. Therefore, the cells only come into contact with formulation that has been diluted to some degree. Only those cells that are in contact with a sufficiently high concentration of formulation are prone to hemolysis. The maximum tolerable concentration that will not produce hemolysis is dependent upon the composition of the formulation. As the plume is diluted, each of the concentration regions becomes more dilute and the hemolytic region becomes smaller. Thus, even though a formulation is hemolytic in the pure state, it may not be hemolytic when injected, especially if it is injected slowly.

If the concentration in the central region of Figure 1a is the minimum concentration that will produce hemolysis, then no hemolysis will be produced by the slow injection depicted. However, since the more rapid injection described by Figure 1b places this hemolytic concentration in contact with a number of cells, it will produce some hemolysis. A more concentrated formulation or a more rapidly injected formulation will produce a greater amount of hemolysis. Within a very short time all of the plume is sufficiently diluted so that it is no longer hemolytic. Therefore, the cells are only in contact with a hemolytic region from immediately after injection until the concentration of the most concentrated region falls below the minimum hemolytic concentration and hemolysis ceases. This is illustrated in Figure 2.

If a plug is formed, the concentrated formulation will have less contact with the cells than if a plume is formed. Note that the very rapid injection rate results in less contact of blood cells with the hemolytic region. This will produce less hemolysis at the injection site. However, as the plug moves downstream and is diluted by blood from intersecting veins, it comes into contact with red blood cells. Although there is less hemolysis at the injection site, there will be more hemolysis downstream.

Precipitation—A number of injectable drugs must be given at a concentration which is higher than their aqueous solubility. The various means of solubilization of drugs and the precipitation of solubilized drugs upon dilution was extensively reviewed by Yalkowsky.[7] The most commonly used means of increasing solubility of injectable drugs is by the addition of a cosolvent or by altering the pH of an aqueous formulation. It was shown that the solubility of many drugs increases exponentially with a linear increase in cosolvent composition or with a linear increase in either hydrogen or hydroxide ion concentration. The concomitant exponential decreases in solubility with a linear decrease in either cosolvent, hydrogen ion, or hydroxide ion is responsible for the fact that many drugs precipitate upon dilution with blood or other aqueous media. When the formulation is diluted to the point at which the concentration of cosolvent, hydrogen ion, or hydroxide ion is not sufficient to maintain the solubility of the drug above the concentration present, precipitation will occur. Valium and Dilantin are among the several commercial products which are highly prone to precipitation upon dilution or injection.

The minimum formulation concentration that can keep the diluted dose in solution is illustrated in the same manner as the minimum hemolytic concentration in Figure 1. For purposes of illustration this is assumed to be the same as the minimum hemolytic concentration. However, these concentrations can differ significantly, depending upon the nature of the drug and the formulation. In this region the drug may form a supersaturated solution, but more likely it will precipitate as either crystals or oil droplets. Precipitated particles or droplets are indicated by "x"s in Figure 1. Note that the minimum hemolytic concentration need not be the same as the minimum precipitation concentration.

If a plug is formed there will be less dilution of the formulation by the blood in the region of the injection site. The only regions in which a supersaturated solution forms are the leading edge and the trailing edge of the plug. Consequently, there will be less precipitation from a very rapid injection than from a slow or moderate rate injection. As the plug becomes further diluted, the concentration of the drug decreases to below the solubility limit, so that no further precipitation will occur.

Phlebitis—Unlike the red blood cells, which flow downstream along with the formulation, the vein wall cells (rectangles in Figures 1 and 2) are stationary and are only in contact with the diluted plume as it passes over them. If the formulation is inherently destructive to the vein wall cells, and if the formulation is sufficiently concentrated, it may cause phlebitis. On the other hand, if the formulation is sufficiently diluted with blood, it will pass over the vein wall cells without producing any effect. However, if the drug precipitates when the formulation is diluted, the crystals will flow downstream with the blood and possibly cause some cell abrasion as they tumble over the inner vein wall. The precipitated particles may also come to rest along the wall of the vein or become embedded in its cells as illustrated in Figure 2. Note that Figure 1 is an enlargement of the left side of Figure 2. In the latter instance some vein wall cells will be in contact with the saturated solution of the drug that is in equilibrium with the precipitate. This causes the cells to be exposed to a very high (mg/kg) dose of the drug. While a nonprec-

Table 1—In Vivo Methods Used To Evaluate Hemolysis after a Single Intravenous Injection

In vivo method	fluid collid	animal model	injection vol (mL)	injection rate (mL/min)	first sample (min)	test soln
Brittain and D'arcy[20]	blood	rabbit	8–12	...	180	cosolvent
Wickliffe et al.[21]	blood	rabbit	5–130	1–10	20	water
Berg et al.[22]	blood	dog	52–156	20	180	water
Benziger[23]	blood	dog	2	0.5	1	cosolvent
Carpenter et al.[24]	blood	dog	10	...	30	cosolvent
Gentry and Black[25]	blood	dog	4.5	2.0	1	cosolvent
Fort et al.[26]	urine	rat	1.0	3.3	180	cosolvent
Fu et al.[27]	urine	rat	0.5	0.17	1440	cosolvent

ipitating formulation (even if saturated) will be in contact with the vein wall cells only momentarily, a precipitate will remain in contact with the vein wall cells until it redissolves. Since redissolution can be quite slow, cell damage from prolonged contact with precipitated drug and its saturated solution can be extensive. Therefore, even if a drug is inherently cytotoxic and its solution will cause phlebitis, precipitation will worsen the phlebitis by prolonging the contact of the drug with the vein wall cells.

Although a number of factors have been implicated as causes of phlebitis, particulate matter is the most clearly documented (Schroeder and DeLuca, 1973). Phlebitis is known to be produced by virtually any particulate matter. Infusion solutions containing very small amounts of particles can produce phlebitis upon prolonged administration. In fact many infusions are given with an in-line filter to minimize the injection of particulate matter. Certainly, precipitated drug, even if it does not adhere to the vein, will act as particulate matter and produce phlebitis.

Pain—Pain is well recognized as a primary symptom of cell damage such as in phlebitis. However, the presence of pain on injection is not indicative of either cell damage or phlebitis. There are a number of types of pain, (e.g., burning, itching, stinging, aching), and none of them can be predicted with any degree of certainty. It is known that pain associated with intravascular injection is usually of short duration unless it is related to phlebitis. This is because the offending substance is rapidly diluted and removed from the injection site. Pain resulting from intramuscular injection is generally of longer duration because the formulation remains in a fairly concentrated form at the injection site for a longer period of time.

In Vivo Studies—To minimize the problems described above for intravenous drug delivery, it is extremely helpful to test all formulations in animals before initiating clinical studies. Of the various animal models available for screening formulations for problems associated with intravenous injection the rabbit ear is the most often used. While any vein of any animal can be used, the rabbit ear vein is the most convenient and reliable. Rabbits are inexpensive compared to dogs, and their ear veins are much more readily accessible and viewable than the veins of other commonly used laboratory animals.

In conducting in vivo studies of intravenous injections it is important to appreciate the concept of effective venous concentration. The effective concentration, EC, of a drug is its concentration in the injected vein at the site of the injection at the time of the injection. Effective concentration has been defined by Ward and Yalkowsky[18] as

$$EC = \text{formulation concentration} \times \frac{\text{injection rate}}{\text{blood flow rate}}$$

Note that a dilute formulation injected rapidly can have the same effective concentration as a concentrated formulation which is injected slowly. It is the effective concentration that indicates the amount of contact of the drug with itself and with various body components. It is therefore the effective concentration that determines whether the formulation will produce precipitation, toxicity to erythrocytes or the cells of the inner wall of the blood vessel, or pain. The need to minimize the effective venous concentration is consistent with the fact that many parenteral manufacturers recommend a slow injection rate for their intravenous products.[18]

In Vivo Hemolysis—Hemolysis can be measured in vivo by analysis of either blood or urine at some time after an intravenous injection. Krzyzaniak[13] reviewed the various attempts at the in vivo quantitation of intravascular hemolysis. The key features of these experimental procedures are summarized in Table 1. Because intravascular hemolysis increases the concentration of circulating free hemoglobin in the blood, the blood level of hemoglobin is a good indicator of hemolysis. Unfortunately, the results of these studies are hard to compare because they are based on different body fluids, different test animals, different injection volumes, different injection rates, and different sampling times, as well as different test vehicles.

Although urine levels of hemoglobin have been used as an indicator of hemolysis, they are much less reliable than blood levels. When the amount of hemoglobin in the plasma is greater than the binding capacity of haptoglobin (a protein which forms a nontoxic complex with free hemoglobin), hemoglobinuria is observed. However when hemolysis is mild, hemoglobin is completely bound to haptoglobin and no hemoglobinuria is observed. On the other hand severe hemolysis can cause renal failure and death.

In Vivo Precipitation—It is possible to physically test for precipitation in vivo by excising the injected ear vein and examining it under polarized light. Powis and Kovach[28] and Sambandan and Yalkowsky[29] used this technique to confirm that bisantrene precipitates in the rabbit ear vein following an intravenous injection. They observed small crystals as well as a bright orange stain (which is characteristic of bisantrene) on the inner wall of the excised vein. Similarly Davis et al.[30] found precipitated diflekirin in monkeys that were given the drug by jugular cannulation.

In Vivo Phlebitis—The most common model for the evaluation of phlebitis is the marginal ear vein of the rabbit. This vein is easily accessible and easily viewed, especially if the ear is shaved. A specified amount of formulation is injected at a specified rate into one vein, and the same vein on the other ear is used as a control. Nearly all early evaluations of phlebitis are based upon a visual comparison of the injected ear with the noninjected control ear. To more precisely quantify phlebitis, many workers have used rating scales similar to that of Table 2. Although these scales are arbitrary and their use is subjective, they provide a means of numerically rating the severity of phlebitis.

There have been a large number of clinical and animal studies in which phlebitis has been evaluated visually by scales such as given in Table 2. Levy et al.[11] evaluated

Table 2—Scale for Visual Evaluation of Phlebitis in the Rabbit[a]

rating	vein color change	region of edema or erythema	inflammation over entire ear
0	no	none	no
1	yes	none	no
2	yes	1–3 mm	no
3	yes	4–8 mm	no
4	yes	≥ 9 mm	no
5	yes	diffuse	yes

[a] Modified from Levy et al.[31] by Ward and Yalkowsky.[21]

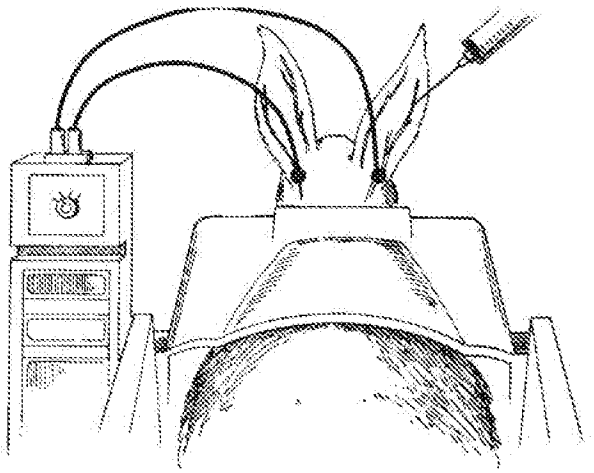

Figure 3—Schematic diagram of experimental setup (from White and Yalkowsky[37] (1991)).

venous sequela produced by a number of diazepam formulations using this scale. Also Ward and Yalkowsky used this scale to evaluate phlebitis produced by intravenous amiodarone and its placebo. Hoover et al.[36] evaluated the venous irritation produced by infusion of each of four antibiotics (tetracycline hydrochloride, erythromycin lactobionate, amphotericin B, and cephaloridine) in the rabbit ear vein. On the basis of a visual analysis of the injection site, they found that the first three produced phlebitis while cephaloridine did not.

While visual evaluation is simple and convenient, it is subjective and somewhat arbitrary. Less subjective and more quantitative in vivo techniques of measuring phlebitis were developed by Ward and Yalkowsky,[21,33,34] Ward et al.,[38,39] and White et al.[37] A specified amount of formulation is injected at a specified rate into the marginal ear vein of a rabbit. The same vein on the other ear is used as a control to account for normal temperature fluctuations. The injected and uninjected ears are monitored for up to 24 h after injection.

Initially a thermal imaging camera was used to measure the increase in temperature of the injected ear vein with respect to the control ear vein. This method provides a noninvasive means for the early detection of phlebitis. Thermal results show good agreement with histological results and with observable symptoms of phlebitis that become evident the next day.[39] More recently an improved method of measuring the temperature increase was developed by Ward et al.[38] and White and Yalkowsky.[37] This method utilizes simple thermocouples, as illustrated in Figure 3, instead of the thermal imaging camera. Thermocouples are placed at a point three centimeters downstream from the injection site and on the corresponding site of the uninjected ear. The injected and control veins are compared visually and thermally over a period of 4 h. A temperature difference of over 2 °C indicates severe phlebitis. An increase of between 1 and 2 °C suggests moderate phlebitis, between 0.5 and 1 °C suggests mild phlebitis, and less than a 0.5 °C suggests no phlebitis. The relationship between an increase in the temperature difference and phlebitis has been confirmed by histological evaluation of both veins after 24 h.[38]

This technique enables the biological evaluation of prototype formulations for their tendency to produce phlebitis. It has been shown to be applicable to diazepam and phenytoin. Using this method, Ward et al.[38,39] and Ward and Yalkowsky[21,33,34] were able to show that an alteration in the marketed formulation of amiodarone (Cordarone, Ayerst) could eliminate phlebitis in rabbits. Similarly, Myrdal et al.[36] and Simamora et al.[40,41] showed that formulations of dexverapamil (the dextro isomer of verapamil) and levomopamil could be easily modified to eliminate phlebitis in rabbits. Also using this method, Simamora et al.[40] showed that buffered placebo solutions ranging from pH 3 to pH 10 do not produce phlebitis.

In Vivo Pain—Unfortunately it is not possible to measure pain in animals without inducing pain. In general an animal will jump and/or vocalize in response to acute severe pain and it will lick or favor an area in response to prolonged mild pain. Therefore, it is possible to at least crudely estimate the intensity of pain in animals by their behavior.

Pain associated with intravenous injection is generally of short duration unless it is due to phlebitis, in which case it can be quite prolonged. Although a rabbit will cry and flinch if given a painful intravenous injection, it is not generally possible to evaluate mild to moderate pain produced by intravenous injection. In fact an animal may not react noticeably to the short duration of moderate pain produced by an intravenous injection. On the other hand, all animals will struggle to get free of a very painful intravenous stimulus. On this basis Marcek et al.[42] developed a novel means of measuring intravascular pain. Rats were restrained and connected to strain gauges. They were given the test solutions 2 h after being cannulated so that there is no effect of the needle stick. The degree of struggling during and following an intravenous injection was taken as a measure of the pain produced by the injection.

Since the pain of an intramuscular or subcutaneous injection tends to last for some time it can be more easily evaluated by the behavior of the animal. For example, Celozzi et al.[43] showed that the number of times a rat or rabbit licks a paw pad in the first minute following a local injection can be taken as a measure of its discomfort. Other workers have used various modifications of this type of test to evaluate formulations. However, it must be realized that any results based upon a prolonged contact between the formulation and the injection site is not relevant to phlebitis.

Alternatively, serum levels of creatine kinase (phosphokinase) could be measured. Creatine kinase is an enzyme that is produced by cells and released in response to damage, and has been used as an indicator of pain on injection by Brazeau and Fung.[44,45] Because this enzyme can be quantitatively measured, it provides a less subjective evaluation of pain. However, it can also be released in response to emotional stress resulting from animal handling and is thus prone to false positive readings.

In Vitro Studies

While in vivo studies provide the most direct measure of the problems commonly encountered with intravenous dosing, they are expensive and time-consuming. They also expose animals to undue stress and pain. Fortunately there are several in vitro studies that can be performed in

Table 3—Summary of Conventional Static in Vitro Hemolysis Studies

authors	formulation–blood ratio	contact time (min)	compounds tested
Husa and Adams[37]	50	30	salts, sugars, and cosolvents
Hartman and Husa[38]	100	45	salts
Grodzki and Husa[39]	100	45	salts and sugars
Rasmussen and Pedersen-Bjergaard[40]	100	45	many pharmaceutical nonisotonic solutions
Cadwallader[41]	100	45	glycerin, propylene glycol
Ansel[42]	200	45	marketed injectable products
Cadwallader and Drinkard[43]	100	45	dimethylsulfoxide
Ku and Cadwallader[44]	100	45	monohydric alcohols
Cadwallader and Phillips[45]	100	45	amides
Smith and Cadwallader[46]	100	45	polyethylene glycols
Oshida et al.[47]	10	30	marketed injectable products
Fort et al.[48]	100	20	ethanol, propylene glycol, PEG-400
Lowe et al.[49]	100	60	surfactants
Al-Assadi et al.[50]	100	20	surfactants
Reiss et al.[51]	1	60	surfactants
Onishi and Sagitani[52]	1	15–120	nonionic surfactants
Rajewski et al.[53]	20	5	cyclodextrins
Shiotani et al.[54]	20	30	complexing agents

lieu of the in vivo studies described above. Since the in vitro studies can be performed by the formulator they are recommended for early formulation development, where they can be used in the selection of a final formulation for toxicology and clinical studies. However, in the interest of human safety, there is no substitute for testing the final formulation in animals (provided, of course, that all in vitro studies indicate that it is safe).

***In Vitro* Hemolysis and Tissue Damage**—The earliest in vitro work on hemolysis concentrated on solution osmolality. However, once Husa and Rossi[38] demonstrated that not all iso-osmotic solutions are isotonic, it became necessary to use red blood cells in screening for hemolytic potential. The first modern method of hemolysis testing was developed by Husa and Adams.[37] They incubated one part blood with fifty parts formulation for fifty minutes, centrifuged to remove the healthy cells and the ghosts of the hemolyzed cells from the supernatant, and then determined the concentration of hemoglobin by colorometric analysis of the supernatant. Following Husa's pioneering work a number of variations on hemolysis testing were proposed and applied to various drugs and excipients. All static in vitro methods of evaluating hemolysis which use a high formulation-to-blood ratio and a long (> 10 min) formulation blood contact time before centrifuging are termed "conventional". Some studies which utilized static methods to determine hemolysis are summarized in Table 3.

Reed and Yalkowsky[14–16] have shown that conventional hemolysis testing gives erratic results when applied to formulations containing certain cosolvents, surfactants, or metal ions because these components alter the ultraviolet spectrum of hemoglobin. Also, Krzyzaniak et al.[5,6] have shown that conventional hemolysis testing (one part blood to ten or more parts formulation and a mixing time which exceeds ten minutes) tends to produce false positive results. The observation of Ansel[42] that marketed formulations of aminophylline, atropine, diphenylhydantoin, and desiccide all produce 100% hemolysis underscores the fact that an exaggerated response is produced by conventional hemolysis methods.

A novel method for the evaluation of the hemolytic potential of drug formulations has been developed by Reed and Yalkowsky.[14–16] Their method involves a "back-titration" in which the cells not hemolyzed by the test solution are separated from the supernatant, washed with normal saline, and then deliberately hemolyzed in distilled water. The hemoglobin released into the water is analyzed spectrophotometrically in the usual manner. The advantage of this method is that the analysis is done in the absence of the components of the test solution which may alter the spectrum of hemoglobin. This method was also used by Fu et al.[37] to determine hemolysis produced by surfactant and cosolvent vehicles.

At the same time Reed and Yalkowsky[14–16] radically changed the formulation–blood ratio from what had been conventionally used. Instead of mixing a small amount of blood with a large amount of formulation, they mixed one part formulation with ten parts blood. This ratio is more representative of the effective concentration produced at the injection site described in Figure 1. They also reduced the formulation blood mixing time from over 10 min (and usually over 30 min) to only 2 min. While this time is not short enough to mimic the rapid dilution of the formulation with blood that is depicted in Figure 2, it was a significant step in that direction.

Oberg and Cadwallader[55] developed a dynamic technique for measuring hemolysis. They were able to produce very short contact times by merging a stream of formulation and a stream of blood in a mixing tube and then quenching the mixture with a large excess of normal saline. The contact time was controlled by the flow rates and by the diameter and length of the mixing tube.

More recently, Krzyzaniak et al.[5,6] have developed a similar dynamic technique for determining the hemolytic potential of formulations at a wide range of contact times. In this method the formulation and blood are mixed in a 1 to 10 ratio before being quenched by a 100 fold excess of normal saline. The mixing time is controlled by the flow rates and the geometry of the mixing tube described in Figure 4. Although they studied a wide range of formulation–blood contact times, they found a 1 s contact time to be most indicative of the situation accompanying an intravenous injection. This method has been shown by Krzyzaniak et al.[6] to give results which are consistent with clinical experience for a number of formulations containing cosolvents and surfactants.

***In Vitro* Models for Precipitation**—The first attempt to quantitate precipitation by an in vitro method was described by Schroeder and DeLuca.[12] Using two syringes they mixed formulation and plasma and passed the mixture through a filter. The amount of precipitation was quantitated by the weight of drug collected on the filter. Using this method they were able to show that the formulation–plasma ratio is a key factor in determining the amount of precipitate that will be formed from a given product. A similar procedure was used by Cox et al.[13]

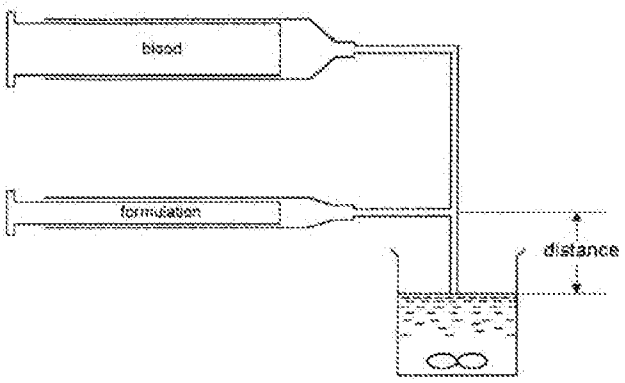

Figure 4—Apparatus for detecting hemolysis at short contact times.

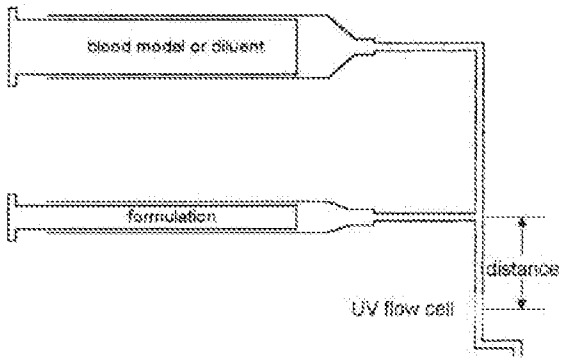

Figure 5—Apparatus for detecting precipitation upon dilution.

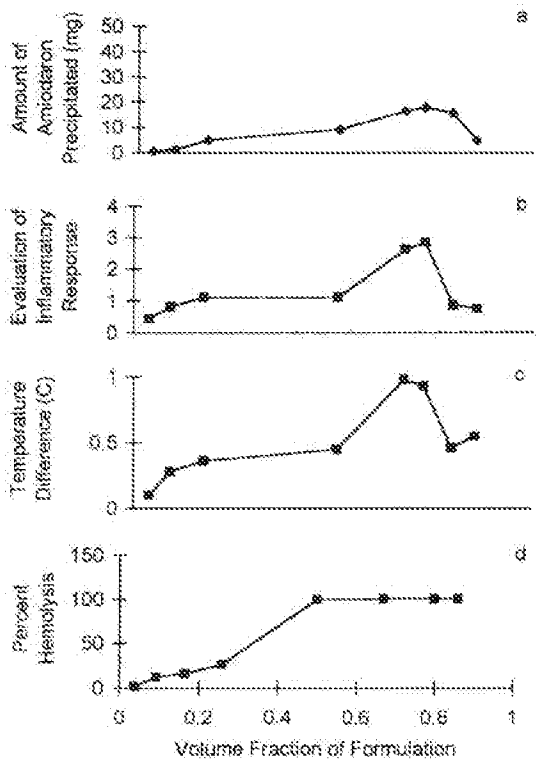

Figure 6—Comparison of (a) precipitation, (b) visual evaluation of phlebitis, (c) thermal evaluation of phlebitis, and (d) hemolysis produced by Amiodarone injection.

A dynamic in vitro method for determining whether a solubilized drug is likely to precipitate when it is injected into a vein or into an intravenous fluid was developed by Yalkowsky and Valvani.[35] Yalkowsky et al.[37] used this method to demonstrate that the amount of precipitation of diazepam injection is inversely proportional to the rate at which it is injected, a fact which is consistent with clinical experience. A given volume of formulation is injected at a specified rate into a stream of Sorensen's phosphate buffer, which is either observed visually or passed through a UV flow cell. The experimental set up is illustrated in Figure 5. Since the absorbance is measured at a wavelength at which neither the drug nor the formulation components absorb, it reflects only opacity, i.e., precipitation is read as absorbance because it interferes with the passage of light through the cell.

This technique enables the determination of the relationship between precipitation and injection rate as described by Yalkowsky.[38] Also, because there is a rough correlation between opacity and the amount of precipitate, this method is semiquantitative. A similar apparatus was used by Irwin and Iqbal[60] for bropirimine and by Singhal[61] for ketoprofen. Cox et al.[62] used this method with plasma as the circulating fluid. When either phenytoin injection or a formulation of a test compound was added to plasma, the opacity decreased due to dilution until precipitation occured after which opacity increased with continued addition as described above.

Using an apparatus similar to that of Figure 5, Davio et al.[30] determined the conditions under which diketorin would not precipitate. Comparison of their in vitro data with data from a 14 day infusion in monkeys demonstrated that "the in vitro precipitation system is useful in establishing concentrations and rates which avoid the problem of intravascular precipitation in preclinical animal studies".[30]

Alternatively a serial dilution of the formulation with equal volumes of Sorensen's phosphate buffer can be used as an indicator of precipitation. A series of 10 tubes provides dilution factors between $\frac{1}{2}$ and $\frac{1}{1024}$. The rate and the amount of precipitation at the various dilution ratios is determined by visual observation. The use of a Tyndall beam can facilitate the observations. Also the cloudy mixtures can be centrifuged or filtered, and the amount of precipitate can be determined from the difference between the concentration of drug in the supernatant and the concentration calculated from the initial concentration and the dilution factor. This technique enables the quantitation of the relationship between precipitation and the degree of dilution as described by Ward et al.[18,31,33,34–36] The results of serial dilution of amiodarone are given in the top portion of Figure 6. This is typical of what is seen for drugs which precipitate on dilution.

Recently Li et al.[7] showed that the dropwise addition of formulation to diluent is a very simple means of assessing the potential of a formulation to precipitate upon dilution. Small fixed quantities (e.g., 0.1 mL) of a formulation are added to a fixed volume (e.g., 10 mL) of either blood or phosphate buffer. After each addition, the mixture is visually examined, preferably with the aid of a Tyndall beam. The appearance of either crystals or cloudiness indicates the minimum formulation–blood ratio that would result in precipitation and is another measure of the dilution stability of the formulation.

Isotonic Sorensen's phosphate buffer is used in the above dilution experiments because it has about the same buffer capacity as in situ blood. Note that neither plasma nor reconstituted blood are used. These do not contain the carbonate concentration of fresh whole blood and thus they do not have the appropriate buffer capacity.

In Vitro Phlebitis—Because there is currently no way to directly model inflammation of the inner vein wall cells in vitro, other cells are used as a surrogate. The most commonly used cells are muscle cells and erythrocytes.

Damage to muscle is measured by the depletion of creatine kinase while damage to erythrocytes is measured by hemolysis.

Hoover et al.[32] showed a relationship between the in vivo rabbit ear phlebitis produced by several antibiotics and their ability to deplete creatine kinase from monkey muscle. Laska et al.[33] showed that creatine kinase depletion of rat muscle cells correlated well with local toxicity for a wide variety of parenterally administered drugs. Brazeau and Fung[34,35] showed a linear relationship between creatine kinase release in injected live rats and in vitro rat muscle cells. They also pointed out the fact that the in vitro use of rat muscle cells eliminates false positive observations resulting from animal stress.

Hoover et al.[32] also suggested that there is a weak relationship between in vitro hemolysis and phlebitis. This is not unreasonable since both hemolysis and phlebitis are the result of cell damage in the region of the injection site. However, Ward and Yalkowsky[36] showed that hemolysis is often not related to phlebitis. Hemolysis is primarily a result of either low tonicity or high local membrane toxicity of dissolved substances in the formulation. In some instances the dissolved substance that is in contact with the vein wall may be present in sufficient concentration and for a sufficient time to cause cell damage. This would result in an apparent agreement between phlebitis and hemolysis. However, this is not likely to be a common occurrence.

The combination of the relationship of injection rate to precipitation and the relationship between injection rate and phlebitis suggests a possible relationship between precipitation and phlebitis. The existence of such a relationship is further supported by the data of Ward and Yalkowsky[36,38] for amiodarone which is shown in Figure 6. These data not only shows that the incidence of phlebitis (as evaluated by either the visual scheme in Table 2 or the thermal method depicted in Figure 3) parallels the increase in precipitation at low to moderate effective concentrations. It also shows that for very fast injection rates there is a decrease in precipitation as well as a decrease in phlebitis (as measured by both methods). This is consistent with the concept of plug flow. The figure also shows that the decrease in precipitation and phlebitis at high injection rates is not paralleled by a decrease in hemolysis. Furthermore, the figure indicates that the amiodarone vehicle does not precipitate nor cause phlebitis at any concentration tested, but it does produce hemolysis at high concentrations.

The lack of a relationship between phlebitis and hemolysis is expected from the description of the injection site in Figures 1 and 2. Phlebitis is usually the result of prolonged contact of precipitated material with the vein wall cells. The importance of prolonged contact is evidenced by the fact reported by Turco[39] that the incidence of phlebitis from solution formulations is increased with increased contact (i.e., infusion) time. Furthermore, if hemolysis were an appropriate model for phlebitis, the latter would not be reduced by a very rapid injection as observed for phlebitis by Ward.[37,38,41]

Phlebitis is believed to be caused primarily by precipitation at the injection site. Therefore, the above precipitation procedures are expected to provide a good indication of the potential of a product to produce phlebitis. However, only an in vivo test will provide a final indication of the effects of precipitation. It will also provide a more realistic indication of the effect of injection rate on phlebitis.

If either precipitation or phlebitis is observed, the formulation can be modified to contain an additional solubilizing agent or additional buffer capacity. Also the formulation can be injected slowly. A slow injection into a constant flowing blood stream produces a rapid dilution. Any concentration of drug becomes soluble as infinite dilution is approached, i.e., any reasonable dose of drug will be soluble in the total blood volume. Therefore, a very slow injection (especially if into a large vein such as the femoral vein) will eliminate precipitation because there will not be sufficient time for nucleation before the drug becomes soluble.

***In Vitro* Models for Pain**—Many parenterals produce pain when injected. Unfortunately, there are no in vitro means of determining whether an intravenous formulation will produce pain. Note that pain resulting from phlebitis can be assumed to be related to the magnitude of the phlebitis which can be modeled by precipitation as described above. Although there is no true in vitro means of assessing pain, cell damage is frequently assumed to be a reasonable indicator. In general either muscle cells or red blood cells are used for the in vitro evaluation of cell damage. While both can be evaluated visually, muscle cells are usually evaluated on the basis of creatine kinase depletion and red blood cells are evaluated by the release of hemoglobin, i.e., hemolysis.

Oshida et al.[37] provided extensive evidence of a correlation between the in vivo muscle damage reported for intramuscular formulations in clinical studies and their in vitro hemolysis. While cell damage may be a reasonable model for pain produced by intramuscular or subcutaneous injections (for which there is prolonged contact of tissue with the undiluted formulation), it is not necessarily applicable to intravascular pain where there is significant dilution of the formulation and a short formulation cell contact time. Hemolysis of cells resulting from a high formulation–blood ratio and/or a prolonged contact time has been proposed by Reed and Yalkowsky[14] as a model for cell damage resulting from intramuscular but not intravascular injection. The inability of hemolysis to consistently model pain is evidenced by the fact that ethanol is only slightly hemolytic at concentrations as high as 95%, while all concentrations above 10% are very painful upon intravenous injection.

Summary

In order for a pharmacological agent to be either effective or toxic it must be in contact with its receptor in sufficient concentration and for a sufficient time to elicit a response. In the case of hemolysis the contact time and concentration at the red blood cell surface are determined by the blood flow rate and the injection rate. A slow injection rate allows the formulation to be rapidly diluted to a concentration which is insufficient to produce hemolysis. Even if the concentration at the injection site is large enough to produce hemolysis, the rapid dilution by blood from other veins might not leave sufficient time for the production of a response. On the other hand if the drug or one of the formulation components precipitates upon dilution with blood, that component may be in contact with the vein wall cells in a high concentration for a prolonged period of time. This can result in phlebitis.

Of the various animal models available for screening formulations for hemolysis and phlebitis the rabbit ear is the most convenient and reliable. However, since all animal use should be minimized, in vitro measurements are more desirable as well as less expensive. Those dynamic in vitro methods that mimic the fluid dynamics at the injection site give results that are more meaningful and which correlate better with in vivo data than do static methods.

While phlebitis is often associated with pain, hemolysis is not. If red blood cells are used as a model for muscle

cells, hemolysis produced by contact with high formulation concentrations for long times can be reflective of pain produced by intramuscular injection. In general those vehicles that produce hemolysis in static testing will also cause tissue damage on intramuscular injection.

In vitro screening of formulations for their potential to precipitate and to produce hemolysis is a simple and inexpensive means of eliminating formulations that are likely to produce clinical problems. Further elimination of unsatisfactory formulations on the basis of in vivo studies using rabbits can then provide a high level of assurance of a successful clinical formulation.

The replacement of hemolytic components by more benign components can produce safer formulations. Precipitation of solubilized drug can be minimized by the use of additional solubilizing agent(s) or additional buffer capacity, provided that they do not produce any additional undesirable effects. The solubilization of drugs for parenteral administration has been discussed extensively by Yalkowsky.[37] If it is absolutely necessary to use a formulation that has the potential to precipitate, phlebitis can be minimized and sometimes totally eliminated by ensuring rapid dilution of the formulation with blood.

Rapid dilution of the formulation, i.e., slow injection and/or deep vein injection, can minimize the above problems by minimizing the effective venous concentrations of the drug and excipients. Any concentration of drug becomes soluble as infinite dilution is approached, i.e., any reasonable dose of drug will be soluble in the total blood volume. A slow injection into a constant flowing blood stream produces a rapid dilution. A slow injection rate and a large blood volume flow contribute to a rapid dilution and a reduced likelihood of precipitation. Therefore, a very slow injection (especially if into a large vein such as the femoral vein) will eliminate precipitation because there will not be sufficient time for nucleation before the drug becomes soluble. Similarly, injection into small veins (such as those of the wrist) should be avoided because their low blood flow rate would result in a high local venous concentration of drug and an increased likelihood of precipitation.

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Some phenomenological and thermodynamic aspects of diffusion in multicomponent systems

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Abstract - During the last twenty years diffusion coefficients have been primarily measured by light scattering and NMR techniques.
Optical interferometric techniques, such as those of Gouy or Rayleigh (ref. 1), allowing direct observation of the time evolution of a diffusing boundary are not very popular at present. However, they are the only ones that give a reasonably accurate measurement of the set of $(n-1)^2$ diffusion coefficients describing the brownian transport process in a multicomponent system.
Experimental data on a variety of ternary systems indicate some aspects of diffusion in multicomponent systems:
(a) The thermodynamic stability conditions:
(i) $D_{11} + D_{22} > 0$ and (ii) $D_{11} D_{22} - D_{12} D_{21} \geq 0$
have been verified experimentally and the relevant contribution of cross terms, which cannot be ignored in describing the transport process, has been pointed out. Furthermore, it was also experimentally verified that on approaching a critical mixing point the determinant (ii) approaches zero.
(b) The main terms need not be necessarily positive; one of them may be negative.
(c) The presence of a binding equilibrium between solutes 1 and 2 affects the experimentally measured values of the four diffusion coefficients.
The equilibrium constant calculated from the experimental D_{ij}'s leads to values in very good agreement with those provided by direct thermodynamic techniques.
The binding equilibrium promotes conditions leading to the transport of one component against its own concentration gradient, or its own chemical potential gradient (passive transport).
(d) Diffusion measurements in three component systems provide a quantitative verification of the effect of the fluid-dynamics equations on the gravitational stability of diffusion boundaries or double diffusive convection, which is a convective transport process of great interest in several fields of pure and applied science.

INTRODUCTION

The aim of this paper is to illustrate briefly some results of the research on brownian diffusion in ternary systems our group has been conducting at the Chemistry Department of University Federico II in Naples for several years.
Its main purpose has been the understanding of the phenomenological role the presence of one component has on the transport process of the others.
The phenomenological diffusion theory proposed by Onsager (ref. 2) describes the transport process in an n-component system by a set of n generalized Fick's equations:

$$(1) \qquad J_i = - \sum_j^n D_{ij} \, \text{grad} \, C_j$$

However, neither the n concentration gradients nor the n flows are all independent.
The relation among concentrations is:

$$(2) \qquad \sum_i^n \bar{V}_i C_i = 1$$

In the absence of pressure gradients, this leads to:

$$(2a) \qquad \sum_i^n \bar{V}_i \, \text{grad} \, C_i = 0$$

where $\bar{V}_i$ is the partial molar volume of component i.

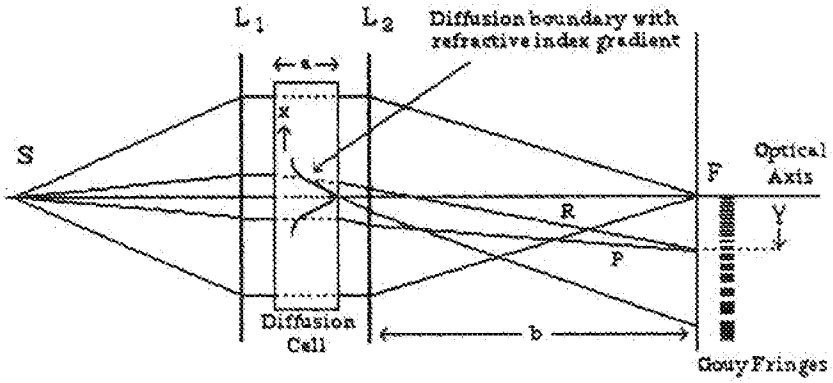

Fig. 1. Scheme of the Gouy Diffusiometer showing the formation of interference fringes. S is the light source, L_1 and L_2 are two lenses that give the source image in F. Light passing through the diffusion boundary, where a vertical gradient of refractive index is present, deviates to form the source image at different levels in F. Light deviation Y, in F, is given by $Y = ab(dn/dx)$, where dn/dx is the refractive index gradient at level x into the diffusion cell. Light wave fronts R and P, passing through the diffusion boundary at different levels, but joining in F, have different optical paths and interfere on the focal plane F to form a fringe pattern.

The relation between flows arises from the continuity law and depends on the reference frame chosen to describe the transport process.
Experimental free diffusion measurements choose a *volume-fixed* reference frame defined by the following relation(ref. 3):

$$(3) \qquad v = \sum_{1}^{n} \bar{V}_i J_i = 0$$

v being the local velocity of a volume element of the solution.
Sometimes it is preferable to describe the flow process in terms of the diffusional velocity of each component ($v_i = J_i/C_i$) with respect to that of component n, generally assumed as the solvent:

$$(4) \qquad J_i = C_i(v_i - v_n)$$

where J_i are the flows in the fixed-solvent reference frame, J_n being zero by definition. A set of n-1 independent equations with a set of $(n-1)^2$ independent coefficients, is obtained by introducing eq.(2) into eqs.(1) :

$$(5) \qquad J_i = -\sum_{1}^{n-1} D_{ij} \operatorname{grad} C_j$$

Onsager suggested the following arbitrary relations to define the n^2 coefficients $\mathcal{D}_{ij}$:

$$(6) \qquad \sum_{j} \mathcal{D}_{ij} C_j = 0 \qquad (i = 1, 2, \ldots n)$$

eq.(6) includes the relation:

$$(7) \qquad \sum_{i,j} \bar{V}_i \mathcal{D}_{ij} C_j = 0$$

A further reduction of independent coefficients arises from the presence of Onsager reciprocity relations. However, their use requires knowledge of the thermodynamic properties of the solution, and this is very limited in multicomponent systems.
Experimental techniques, such as the Gouy interferometer (ref. 1) used in our research (see Fig. 1), give the $(n-1)^2$ diffusion coefficients of a n-component system, at one mean concentration, from the analysis of the diffusion patterns of at least (n-1) diffusion runs with varying concentration differences of their components through the diffusion boundary. This technique has so far been employed only in the investigation of three component systems.

CHOICE OF COMPONENT *n* (the solvent)

In a 3-component system, equations (5) reduce to:

$$(8) \qquad J_1 = -D_{11} \operatorname{grad} C_1 - D_{12} \operatorname{grad} C_2$$
$$(8) \qquad J_2 = -D_{21} \operatorname{grad} C_1 - D_{22} \operatorname{grad} C_2$$

and four diffusion coefficients can be measured experimentally.

The flow of component 3 is obtained from equation (3):

$$(9) \quad J_3 = \left(\frac{\bar{V}_1 D_{11} + \bar{V}_2 D_{21}}{\bar{V}_3}\right) \text{grad}\, C_1 + \left(\frac{\bar{V}_1 D_{12} + \bar{V}_2 D_{22}}{\bar{V}_3}\right) \text{grad}\, C_2$$

However, it must be pointed out that the choice of component 3, commonly defined as the solvent, is arbitrary. In general it is the most abundant or that weighed last in preparing solutions for diffusion measurements. Equations (8) can be written in terms of grad C_1 and grad C_3 or grad C_2 and grad C_3, as well.

Depending on the choice of the concentration differences (ΔC_i and ΔC_j) between bottom and top solutions used in elaborating the experimental results of diffusion runs, three sets of four diffusion coefficients are obtained.

Let us define $(D_{ij})_k$ the diffusion coefficient of component i under the concentration gradient of component j in a ternary system where component k has been chosen as solvent (where not necessary index k is omitted). The following transform expressions correlate the three sets of diffusion coefficients (ref. 3):

$$(10a) \quad (D_{ii})_j = (D_{ii})_k - (\bar{V}_i/\bar{V}_j)(D_{ij})_k$$

$$(10b) \quad (D_{ik})_j = -(\bar{V}_k/\bar{V}_j)(D_{ij})_k$$

$$(10c) \quad (D_{ki})_j = (\bar{V}_i/\bar{V}_k)[-(D_{ii})_k + (\bar{V}_i/\bar{V}_j)(D_{ij})_k - (\bar{V}_j/\bar{V}_i)(D_{ji})_k + (D_{jj})_k]$$

$$(10d) \quad (D_{kk})_j = (D_{jj})_k + (\bar{V}_i/\bar{V}_j)(D_{ij})_k$$

From eqs.(10) it can be seen that the determinant and the trace of matrices $(\mathbf{D})_k$ are invariants.

Although the transport process is a single one and independent of the choice of component 3, this choice may help to a better understanding of the diffusion process. An example is given by the flow equations of the system:

Sucrose (0.097 M)(1) - Sodium Chloride (0.291 M)(2) - Water (53.93 M)(3)

at 25°C (ref. 4) (in eq. (11) and (12) diffusion coefficients units are in 10^5 cm^2 s^{-1}):

$$(11) \quad J_1 = -0.487 \text{ grad } C_1 + 0.002 \text{ grad } C_2 \qquad \text{(component k} \rightarrow \text{water)}$$

$$(11) \quad J_2 = -0.087 \text{ grad } C_1 - 1.475 \text{ grad } C_2 \qquad \text{"} \qquad \text{"}$$

$$(12) \quad J_1 = -0.512 \text{ grad } C_1 - 0.002 \text{ grad } C_3 \qquad \text{(component k} \rightarrow \text{NaCl)}$$

$$(12) \quad J_3 = -10.92 \text{ grad } C_1 - 1.450 \text{ grad } C_3 \qquad \text{"} \qquad \text{"}$$

As eqs. (11) show, the sucrose(1) - NaCl(2) - water(3=k) system has small cross diffusion coefficients, one of them is almost zero. However, things are quite different when NaCl is chosen as component k. The new $(D_{ij})_k$ set shows that the water cross diffusion coefficient is one order of magnitude larger than its main term. This indicates that water flows much faster in the sucrose concentration gradient than in its own, and that it can even flow against its own concentration gradient within the following concentration gradients:

$$\text{grad } C_3 < -7.53 \text{ grad } C_1 > 0$$

The water behaviour, although hidden in eqs.(11), is clearly shown only by eqs. (12).

In general, the cross diffusion coefficients cannot be ignored in describing the transport process. Large and positive D_{ij} values have been observed in systems where the solutes tend to *salt-out* (ref. 4). Large negative cross-term diffusion coefficients can occur in systems with large attractive interactions between solutes (ref. 4-6). The data shown in Table 1 confirm this statment.

Table 1 collects the $(D_{ij})_k$ for the **Acetic acid- Chloroform- Water** system at various compositions, the data for all three choices of component k are given. It can be seen that if components i and j are water and chloroform (salting out effect) the cross terms are positive; if they are water and acetic acid (attractive interactions), the cross terms are negative.

TABLE 1. Diffusion Coefficient for the System: Acetic Acid-Chloroform-Water at 25°C (ref. 7) and various compositions. X_i = mol fraction of component i. D units 10^5 $cm^2\,s^{-1}$

Component i → Acetic Acid; Comp. j → Chloroform; Comp. k → Water					
X_i	X_j	$(D_{ii})_k$	$(D_{ij})_k$	$(D_{ji})_k$	$(D_{jj})_k$
0.8803	0.0496	1.296	0.011	-0.232	0.933
0.7811	0.0892	1.550	0.520	-0.548	0.367
0.6924	0.1288	1.547	0.545	-0.624	0.230
0.5405	0.1897	1.814	1.065	-0.939	**- 0.334**
0.4199	0.2400	1.844	1.153	-1.095	**- 0.596**

Component i → Water; Comp. j → Chloroform; Comp. k → Acetic Acid.					
X_i	X_j	$(D_{ii})_k$	$(D_{ij})_k$	$(D_{ji})_k$	$(D_{jj})_k$
0.0701	0.0496	0.970	0.130	0.073	1.259
0.1297	0.0892	0.782	0.194	0.172	1.135
0.1788	0.1288	0.672	0.236	0.196	1.105
0.2698	0.1897	0.498	0.321	0.295	0.983
0.3401	0.2400	0.309	0.368	0.344	0.939

Component i → Water; Comp. j → Acetic Acid; Comp. k → Chloroform					
X_i	X_j	$(D_{ii})_k$	$(D_{ij})_k$	$(D_{ji})_k$	$(D_{jj})_k$
0.0701	0.8803	0.941	-0.093	-0.002	1.288
0.1297	0.7811	0.738	-0.138	-0.117	1.178
0.1788	0.6924	0.619	-0.168	-0.122	1.158
0.2698	0.5405	0.426	-0.229	-0.239	1.055
0.3401	0.4199	0.226	-0.262	-0.258	1.022

THERMODYNAMIC STABILITY CONDITIONS

Thermodynamic stability is ensured by the condition that any perturbation promotes an entropy absorption; namely, the entropy at equilibrium is a maximum; on the other hand, any spontaneous process occurs with a positive definite entropy production.
For isothermal diffusion in an n-component system this condition is written as:

$$(13) \qquad T\delta^2 S = -\sum_i \delta\mu_i\,\delta n_i \le 0 \qquad \text{where} \quad \delta\mu_i = \sum_k \frac{\partial \mu_i}{\partial n_k}\,\delta n_k$$

$\delta\mu_i$ being the perturbation of chemical potential of component i, and δn_k the possible arbitrary fluctuation in the number of moles of component k.
From eq.(13) one obtains the stability conditions with respect to diffusion:

$$(14) \qquad \sum_{i,k} \mu_{ik}\,\delta n_i\,\delta n_k \ge 0 \qquad \text{where} \quad \mu_{ik} = \frac{\partial \mu_i}{\partial n_k}$$

The conditions for the quadratic expression (14) to be positive or zero are that all μ_{ii} must be positive and all the other minors, both odd and even order, constructed on the principal diagonal of the determinant of μ_{ik} must be positive or zero. This condition is met by making the trace of the matrix of diffusion coefficients positive and the determinant positive or zero. In a ternary system:

$$(15) \qquad D_{11} + D_{22} > 0 \quad \text{and} \quad D_{11}\,D_{22} - D_{12}\,D_{21} \ge 0$$

The equality sign corresponds to the boundary between stable (or metastable) and unstable systems. In the phase diagram this boundary is known as the spinodal curve. Any solution whose composition lies within this curve is unstable and concentration fluctuations always promote a phase separation.
The equality sign was confirmed in a set of experimental diffusion runs on the water-chloroform- acetic acid system (ref. 7) taken at various compositions approaching the plait-point, where the phase separation curve joins the spinodal curve; the results are shown in Fig. 2.
Conditions (15) do not require both main diffusion terms to be positive. Diffusion coefficients measured in a volume-fixed reference frame may give a negative main term. This was found in the system shown in Fig. 2. Table 1 collects the set of $(D_{ij})_k$ obtained choosing each component as component k in turn.
It can be seen that when water is chosen the chloroform main diffusion coefficient is negative in a wide range of compositions approaching the plait- point.

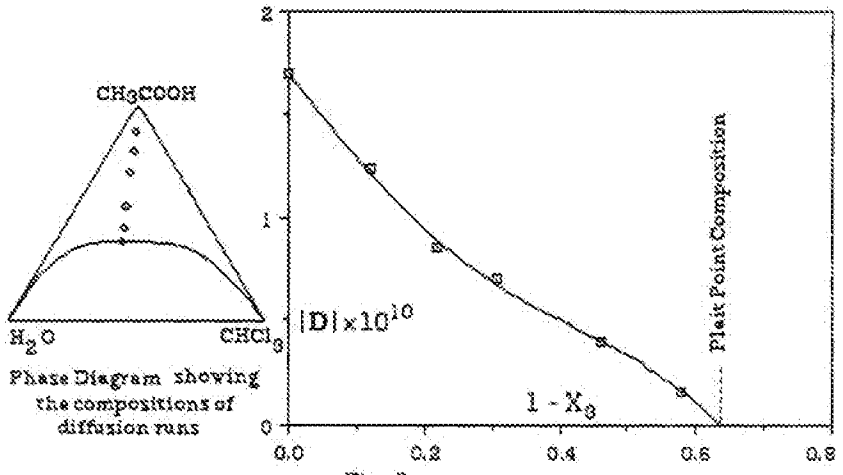

Fig. 2.

Fig. 2. System Acetic acid - Chloroform - Water at 25°C (ref. 7); |**D**| Determinant of Diffusion Coefficients ; X_3 mole fraction of acetic acid

DIFFUSION IN THE PRESENCE OF A BINDING EQUILIBRIUM

While the size and sign of diffusion coefficients for systems with high solute concentrations are only understood in qualitative terms at present, a more detailed interpretation of data is possible for dilute solutions in which significant fractions of solute monomer species have combined to form associated species.
Let us consider a solution of monomer species A (1) and B (2) in equilibrium with a complex AB (3) according to the equation:

(16) $A + B = AB$

This is a quaternary system and diffusion eqs.(5) require nine coefficients, D_{ij}^* (i, j = 1, 2, 3). However, if we are dealing with a dilute solution of both A and B, the cross diffusion terms are expected to be small as compared to the main terms, and they can reasonably be ignored in the flow equations (5).
Furthermore, in the presence of a fast equilibrium, the mass conservation law:

(17) $C_1 = C_1^* + C_3^*;\ C_2 = C_2^* + C_3^*;\ J_1 = J_1^* + J_3^*;\ J_2 = J_2^* + J_3^*$

(where C_i and J_i are the stoichiometric concentration and flow of component i, i = 1, 2, and C_j^* and J_j^* are the concentration and flow of the actual species j, j = 1, 2, 3) and the mass action law:

(18) $K_c = C_3^* / (C_1^* C_2^*)$

impose a restriction between concentrations and flows.

Because of eqs.(17) and (18) this system reduces to a ternary one having only four measurable diffusion coefficients which are related to the actual diffusion coefficients D_{ij}^* by the following expressions:

$$(19a) \quad D_{11} = (1/2)\{(D_{11}^* + D_{33}^*) + (D_{11}^* - D_{33}^*)[1 - K_c(C_2 - C_1)]\, R\}$$

$$(19b) \quad D_{12} = (1/2)\{(D_{33}^* - D_{11}^*) + (D_{11}^* - D_{33}^*)[1 + K_c(C_2 - C_1)]\, R\}$$

$$(19c) \quad D_{21} = (1/2)\{(D_{33}^* - D_{22}^*) + (D_{22}^* - D_{33}^*)[1 - K_c(C_2 - C_1)]\, R\}$$

$$(19d) \quad D_{22} = (1/2)\{(D_{22}^* + D_{33}^*) + (D_{22}^* - D_{33}^*)[1 + K_c(C_2 - C_1)]\, R\}$$

where:

$$(20) \quad R = \left([\, 1 + K_c\,(C_2 - C_1)]^2 + 4K_c C_1 \right)^{-\frac{1}{2}}$$

Eqs. (19) are a set of four equations allowing the three D_{ij}^* and K_c at each concentration to be computed from the experimental D_{ik}.

Eqs.(19-20) were verified for (a) **α-cyclodextrin-(L)phenyl alanine- water** (ref. 8,9) and (b) **α-cyclodextrin-(D,L)norleucine-water** (ref. 10) at 25°C and various amino acid concentrations. The K_c obtained from eqs.(19-20) was in very good agreement with that measured calorimetrically. Table 2 collects the diffusion data for (a) and (b) at one concentration and the average K_c from the diffusion and the calorimetry data (ref. 8-10).

TABLE 2. Diffusion data for (a) α- cyclodextrin(1) - (L)phenyl alanine(2) - water and (b) α-cyclodextrin(1) - (D,L)norleucine(2) - water at 25°C (ref. 8-10):

(a) $C_1 = 0.0200$ mol/L, $C_2 = 0.1000$ mol/L, $K_c = 10.5$ L/mol, $K_{cal} = 13.6$ Kg/mol*
(b) $C_1 = 0.0198$ mol/L, $C_2 = 0.0602$ mol/L, $K_c = 49$ L/mol, $K_{cal} = 46$ Kg/mol*
* In dilute aqueous solution MOLAR and MOLAL equilibrium constants do not differ appreciably

	D_{11}	D_{12}	D_{21}	D_{22}	(10^6 cm^2 s^{-1})	D_{11}^*	D_{22}^*	D_{33}^*
(a)	3.157	0.000	-1.630	6.332		3.157	6.510	3.157
(b)	3.142	-0.003	-2.178	6.270		3.163	6.432	3.128

The inclusion equilibrium drastically affects the value of the cross diffusion term responsible for aminoacid (A) transport under the concentration gradient of cyclodextrin (CD).

As can be seen from eq.(19b), the near equality of D_{11}^* and D_{33}^* is responsible for the very small cross-term diffusion coefficient D_{12}.

The cross-term D_{21} tends to be large and negative because the diffusion coefficient D_{22}^* of the faster moving A is much larger than D_{33}^* for the CD-A complexes. In this case, if there is a CD gradient and no gradient of A, the negative D_{21} causes A to move *uphill* towards the higher concentration of CD. This is because a higher concentration of CD means a lower concentration of unbounded A.

The transport of one component due to the concentration gradient of the other (passive transport) bears some similarity to the active transport that plays a substantial role in biological membrane processes.

Passive transport may also be of interest in membrane processes, the presence of cross terms can in fact lead to conditions for which one component diffuses against its own concentration gradient or even its chemical potential gradient, under the driving force of the other one.

GRAVITATIONAL INSTABILITIES / IN FREE DIFFUSION BOUNDARIES

In recent years, there has been a great interest in *double diffusive convection*, i.e. the convective motions which can arise in diffusive layers even if the top liquid system has a lower density than the bottom one. This phenomenon may appear in systems where at least two independent driving forces promote the transport process. Thus, it may appear in binary solutions when a temperature and a concentration gradient are present, or in isothermal ternary solutions in the presence of concentration gradients of two components (ref. 11).

An example is the heat and salt diffusion that gives rise to *fingering* or *layering* in oceans. Fig. 3 shows the temperature and salt concentration gradient conditions that may promote the growth of two different convective mechanisms in salty water.

Other examples include unwanted convection in lakes and solar ponds, rollover in liquid natural gas tanks, geology (crystallization and magma chamber processes), geophysics (mantle convection and vulcanism), astrophysics (inside stars at least 4 components may be involved in the process: angular momentum, heat, magnetic field, helium / hydrogen composition), metallurgy (morphology and crystallization).

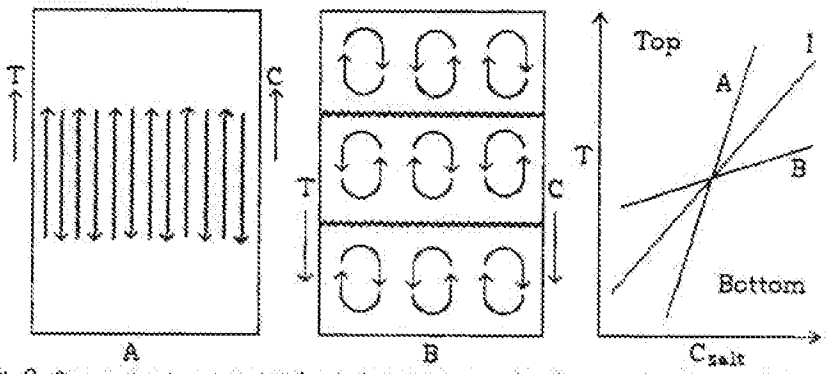

Fig. 3. Convection in sea water due to temperature and salt concentration gradients: I, isodensitmetric line. A, Fingers growth. B, Formation of convection cells.

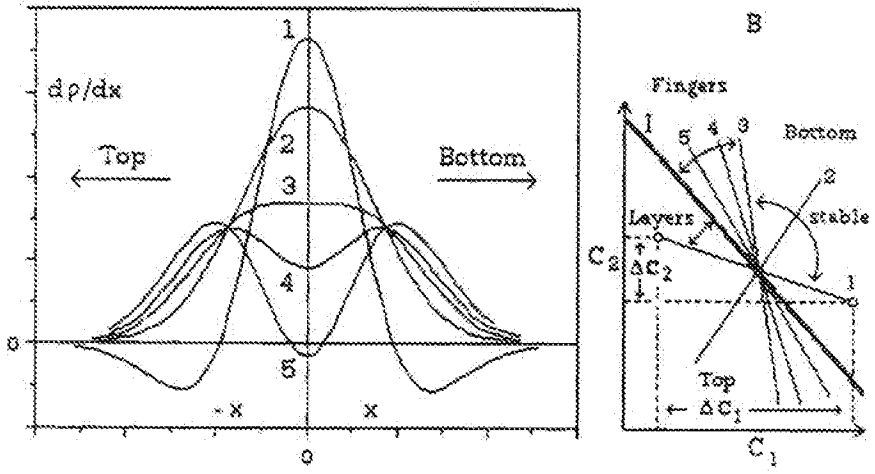

Fig. 4. Qualitative graph showing the density gradients through a diffusion boundary for various $\Delta C_2 / \Delta C_1$ conditions. B: graph showing the average composition of a set of diffusion runs, the concentration differences between bottom and top solutions, and the fields of stable and unstable boundaries. 1- convection at the borders of the boundary (*layering*). 2 - stable boundary. 3,4 - convection at the center of the boundary (*fingers*) with no density inversion inside the boundary due to diffusion. 5 - density inversion at the center of the boundary due to diffusion. I - isodensitmetric line.

Free diffusion experiments can be performed in well-controlled conditions and have provided an accurate way of testing the fluid-dynamics theories concerned (ref. 4,12-14).

The profile of density (ρ) gradient through the diffusion boundary is given by the sum of two gauss functions (ref.15). Its shape depends on the ΔC_1 and ΔC_2 concentration differences of components 1 and 2 across the diffusion boundary, and on the diffusion coefficients. Two different kinds of instabilities were observed in free diffusion boundaries:
(a) A dynamic instability arising at the center of the boundary, even in the absence of density inversions due to the diffusion process [Fig. 4 (3, 4)], for:

$$(\partial^2 \rho/\partial x^2)(1/x) \leq 0 \qquad (21)$$

In this case convection tends to destroy the boundary and the process is similar to the *fingering* in sea water (Fig. 3 A).
(b) A static instability, namely a density inversion, at the borders of the boundary, as shown in Fig. 4(1). In this case convection at the borders of the boundary promotes its apparent overstabilization and the diffusion boundary keeps itself sharp and narrow much longer than expected from the diffusion experiment (ref. 16). This process is similar to the layering effect in sea water, shown in Fig. 3 B.

Acknowledgement

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Formulation Development and Antitumor Activity of a Filter-Sterilizable Emulsion of Paclitaxel

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Purpose. Paclitaxel is currently administered i.v. as a slow infusion of a solution of the drug in an ethanol:surfactant:saline admixture. However, poor solubilization and toxicity are associated with this drug therapy. Alternative drug delivery systems, including parenteral emulsions, are under development in recent years to reduce drug toxicity, improve efficacy and eliminate premedication.

Methods. Paclitaxel emulsions were prepared by high-shear homogenization. The particle size of the emulsions was measured by dynamic light scattering. Drug concentration was quantified by HPLC and *in vitro* drug release was monitored by membrane dialysis. The physical stability of emulsions was monitored by particle size changes in both the mean droplet diameter and 99% cumulative distribution. Paclitaxel potency and changes in the concentration of known degradants were used as chemical stability indicators. Single dose acute toxicity studies were conducted in healthy mice and efficacy studies in B16 melanoma tumor-bearing mice.

Results. QW8184, a physically and chemically stable sub-micron oil-in-water (o/w) emulsion of paclitaxel, can be prepared at high drug loading (8–10 mg/mL) having a mean droplet diameter of <100 nm and 99% cumulative particle size distribution of <200 nm. *In vitro* release studies demonstrated low and sustained drug release both in the presence and absence of human serum albumin. Based on single dose acute toxicity studies, QW8184 is well tolerated both in mice and rats with about a 3-fold increase in the maximum-tolerated-dose (MTD) over the current marketed drug formulation. Using the B16 mouse melanoma model, a significant improvement in drug efficacy was observed with QW8184 over Taxol®.

Conclusions. QW8184, a stable sub-micron o/w emulsion of paclitaxel has been developed that can be filter-sterilized and administered i.v. as a bolus dose. When compared to Taxol®, this emulsion exhibited reduced toxicity and improved efficacy most likely due to the composition and dependent physicochemical characteristics of the emulsion.

KEY WORDS: paclitaxel; emulsions; filter-sterization; particle size; stability

INTRODUCTION

Paclitaxel is an important chemotherapeutic agent with a wide spectrum of activity against solid tumors primarily breast, ovarian, colon and non-small cell lung carcinomas (1). The drug exerts its antitumor activity by binding to tubulin and stabilizing microtubules and thus blocking cell mitosis (2). Paclitaxel is a natural product present in the bark of the Pacific Yew tree and like other natural products has limited aqueous solubility (3). The drug is only administered intravenously since it is orally inactive due to membrane transport and liver metabolism limitations (4).

The commercially available product, Taxol® (paclitaxel injection, Bristol-Myers Squibb Oncology), is currently formulated in a vehicle containing approximately a 1:1 v/v mixture of polyoxyethylated castor oil (Cremophor EL) and ethanol. Cremophor EL, a commonly used surfactant for lipophilic compounds, has been associated with bronchospasms, hypotension, and other manifestations of hypersensitivity particularly following rapid administration (5,6). Long infusion times upon a 10-fold dilution of the ethanol:Cremophor EL solution and premedication are therefore required to reduce these adverse effects. Furthermore, this formulation is associated with a number of issues such as stability with the possibility for drug precipitation upon dilution, filtering requirements and use of non-plasticized containers and administration sets (6). It is thus apparent that there is a need for new formulations of paclitaxel that are efficacious and less toxic than the commercial product and can alleviate drug administration issues.

In recent years considerable emphasis has been given to the development of new formulations of paclitaxel that are suitable for intravenous administration to address the aforementioned drug solubility and toxicity issues. These include dispersed systems such as emulsions (7–11), liposomes (4,12,13), mixed micelles (14), cyclodextrins (15) and microspheres (16). Water-soluble prodrugs such as polyethylene glycol- and polyglutamate-paclitaxel with promising antitumor activity have also been developed (17,18).

An o/w emulsion of paclitaxel using triacetin and ethyl oleate as the oil phase, lecithin and pluronic F-68 as the surfactant has been reported earlier by Tarr et al. (7). Although high levels of paclitaxel have been solubilized in this emulsion (10–15 mg/mL), no antitumor activity was reported. This is due to slow precipitation of the drug upon dilution with a dextrose solution and toxicity of triacetin at concentrations required for delivering therapeutic doses of paclitaxel, with a reported LD_{50} of 1.2 mL/kg (7).

Oil-in-water emulsions using safflower or soybean oil, lecithin and cholesterol and incorporating paclitaxel up to 5 mg/mL have been reported by Kaufman et al. (8). The mean droplet diameter of these emulsions was reported to remain between 0.2 to 0.4 μm upon a six-week storage at 40°C. An o/w emulsion of paclitaxel and Taxol® were evaluated for toxicity in rats upon dilution to 1 mg of paclitaxel per mL followed by i.v. infusion over 30 min. At 42 mg/kg (42 mL/kg of the diluted formulation) there were signs of severe toxicity in the animals treated with Taxol® whereas animals treated with the emulsion showed low toxicity that was manifested primarily as a body weight loss (8). When the emulsion and the Taxol® formulation were evaluated *in vitro* for efficacy against mouse lymphocytic leukemia (L1210) or rat mammary adenocarcinoma (NMU) cell lines, similar antitumor activity was observed (8).

Lundberg (9) reported on the preparation and evaluation of complex submicron paclitaxel emulsions incorporating triglycerides, phospholipids, polysorbate 80 and pegylated phospholipids to prolong the circulation of the emulsion particles

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in the blood. These emulsions, triolein:dipalmitoylphosphatidylcholine: polysorbate 80: PEG-phosphatidylethanolamine: paclitaxel (1:1:0.4:0.1:0.03, mass ratio) with a mean droplet diameter of about 40 nm (9) showed good physical and chemical stability at low temperature and/or as a lyophilized powder. Paclitaxel-loaded emulsions demonstrated good antitumor activity (*in vitro*) when compared to Taxol®. No *in vivo* studies were reported.

Recently, some prototype o/w emulsions using benzyl benzoate and tributyrin as the oil phase, Tween 80 and Arlacel 20 as surfactants and incorporating up to 5 mg/mL paclitaxel have been reported by Simamora et al. (10). Although these emulsions were found to be stable upon dilution with a variety of i.v. fluids with no evidence of local irritation, studies on vehicle toxicity and drug efficacy have not been reported.

The objective of this study was to develop an injectable emulsion formulation of paclitaxel using vitamin E as the oil phase and having the following characteristics: a) incorporate high levels of paclitaxel (8–10 mg/mL) and is physically and chemically stable; b) has mean droplet diameter and 99% cumulative distribution of less than 0.2 μm and thus can be filter-sterilized, and c) less toxic and at least as efficacious as the commercial formulation Taxol®. The use of vitamin E as the oil phase in the present emulsions may have beneficial effects in reducing drug toxicity since vitamin E has been reported to be therapeutic of mucositis (19), one of the principal side effects of Taxol® therapy. Vitamin E is widely reported to reduce the generalized toxicity of other cytotoxics (20).

MATERIALS AND METHODS

Materials

Vitamin E (DL-α-tocopherol) USP/FCC grade was purchased from Roche (Belvidere, NJ). Easiman's vitamin E-TPGS (α-tocopherylpolyethyleneglycol-1000 succinate) was supplied by B&D Nutritional Ingredients, Inc. (Carlsbad, CA). Polyethylene glycol 400 N.F. (PEG 400) and Poloxamer 407 (Pluronic F-127) were purchased from Spectrum Quality Products, Inc. (Gardena, CA) and BASF Corporation (Mount Olive, NJ), respectively. Paclitaxel (>99% purity) was supplied by Hauser Laboratories (Boulder, CO) and Hande Tech. Development Co. (Houston, TX) and stored desiccated. Taxol®, 5 mL vial at 6 mg/mL, was purchased from a pharmacy and stored refrigerated.

Particle Size

Mean droplet diameter and particle size distribution were determined with a Nicomp 370 Submicron Particle Sizer using a 5 mW laser beam at 632.8 nm (Particle Sizing Systems, Santa Barbara, CA). Polystyrene bead standards were used to verify the calibration of the instrument. Data was analyzed in terms of intensity, volume and number distributions and reported here as volume weighted distribution.

Emulsion Preparation

A pre-emulsion was first prepared by adding the oil phase (α-tocopherol) in PEG 400 containing the surfactants (TPGS and Poloxamer 407) with or without paclitaxel to degassed water with vigorous mixing at 45°C followed by homogenization in the Avestin Emulsiflex C-5 (20–200 mL batches) or Emulsiflex C-50 (0.5–3.0 L batches) high-pressure homogenizers (Ottawa, Canada). Following homogenization, the finished product was terminally sterilized by filtration through a 0.2 μm Posidyne filter (Pall Corp., East Hills, NY). The resulting emulsions contained (%, w/w): α-tocopherol, TPGS (1–10%), Poloxamer 407 (1–5%), PEG 400 (1–10%), paclitaxel (0.5–1.0%) and water for injection (60–90%).

Physical and Chemical Stability

Emulsions were analyzed for paclitaxel content by high performance liquid chromatography (21) on a Phenosphere CN column (5 microns, 150 × 4.6 mm). The mobile phase consisted of a methanol/water linear gradient beginning with 40:60 mixture (v/v) at a flow rate of 1.0 mL/min and reaching 100% methanol after 15 minutes. A UV detector at 227 nm was used to detect and quantitate paclitaxel. A single peak was detected which had a retention time and mass spectrogram consistent with reference paclitaxel obtained from Hauser Laboratories (Boulder, CO).

Emulsions were stored at 4°C or 25°C and samples were removed at predetermined time points and assayed for physical and chemical stability. The mean droplet diameter and 99% cumulative distribution of the particles were used as indicators of physical stability. Paclitaxel potency and the change in concentration of the known degradants were monitored for chemical stability. The following degradants were monitored during the stability program: 7-epi-paclitaxel, 10-deacetyl-paclitaxel and baccatin III.

Drug Release

In vitro release of paclitaxel from different formulations in the presence and absence of human serum albumin was monitored by membrane dialysis at 37°C (22) using phosphate-buffered saline as a sink solution at pH 7.0. The concentration of paclitaxel in Taxol® and QW8184, injectable paclitaxel emulsion, was 6.0 and 8.8 mg/mL, respectively, and the drug to albumin molar ratio was 1000 to 1. The sample volume in the dialysis bag (cassette) was 1 mL and the sink volume 500 mL. Assuming 100% drug release, the concentration of paclitaxel in the sink solution will be <20 μg/mL which is within the reported aqueous paclitaxel solubility range of 1–40 μg/mL (9). Pierce Slide-A-Lyzer® dialysis cassettes with a MW cutoff of 10K, and thus freely permeable to paclitaxel (MW = 853), were used and temperature control was maintained with jacketed beakers via circulating bath. Concentrations of the drug in pre-/post-dialysis samples and aliquots at various time intervals were determined and drug release profiles (% drug released vs time) were generated.

Single Dose Acute Toxicity

The maximum tolerated dose (MTD) of paclitaxel emulsions was determined in mice following a tail vein injection using 3–6 animals per group. Emulsions were administered i.v. at bolus doses ranging from 20–90 mg/kg or 2–10 mL/kg of vehicle. Taxol® was first diluted with saline and then administered as 1–2 minute i.v. infusion at doses ranging from 5–25 mg/kg. A saline control group was also included. The determination of the MTD was based on body weight loss (>15%) and mortality, i.e. number of deaths per group (23,24).

Efficacy: B16 Melanoma

Female B6D2F mice (8 mice per group) were subcutaneously (sc) implanted with 10^7 B16 melanoma tumor cells (23,24). Four days after implantation, mice were randomly sorted into treatment groups and were administered i.v with saline, Taxol®, or QW8184 emulsion with and without incorporated paclitaxel on a schedule of either q3d × 5 or q4d × 5, that is, once every three or four days, respectively, for a total of 5 doses. QW8184 was administered as a bolus injection and Taxol® was infused over 2 minutes following 10-fold dilution with saline. The administered volume of saline and drug-free emulsion (vehicle) were 7 and 7 or 8 mL/kg, respectively. For specific dosages and schedules see the Figs. 4 and 5 legends. Antitumor activity was assessed according to the guidelines established by the National Cancer Institute (24).

RESULTS AND DISCUSSION

Development of Filter-Sterilizable Emulsions

The formulation development strategy of using α-tocopherol as the oil phase and TPGS as the primary surfactant was followed to develop a stable and efficacious emulsion of paclitaxel at high drug loading that can be filter-sterilized. Ensuring product sterility of parenteral emulsions is crucial and terminal heat sterilization has been generally used for this purpose. Alternatively, certain emulsions can be lyophilized and reconstituted prior to administration without loss of viability and drug potency (25). If, however, the components of a particular drug-emulsion are heat labile, filter sterilization of the product may be a viable option, provided that the emulsion droplets pass through 0.2 micron pore. However, filter sterilization of emulsions is quite challenging and use of this non-invasive method of sterilization has been limited (26).

Several prototype formulations were developed which, upon further optimization, yielded the lead development candidate QW8184. Formulation optimization was based on the use of an alternate co-surfactant or co-solvent, and the resulting emulsions were evaluated for acute and cumulative toxicity, filterability, drug loading, and where appropriate, stability and efficacy. Several of the prototype formulations were found to be stable and efficacious; however, only data with the most advanced formulation tested to date, QW8184, is presented.

Physical and Chemical Stability

Physical stability of an emulsion is one of the most important desired product characteristics. Emulsions are heterogeneous systems and thermodynamically unstable and, therefore, have a significant tendency to lose physical stability on storage. The extent of this process is dependent on the characteristics of each formulation and storage conditions. Some of the factors that affect the physical stability of emulsions include the type and levels of surfactant(s) used to stabilize the droplets, the phase volume ratio, droplet size, compatibility of drug and excipients with the emulsion, and storage conditions of the emulsion (25,27). In general, the smaller the particle size of the emulsion, the better its physical stability (25,27). The assessment of the physical stability of emulsions and of dispersed systems in general, in addition to being extremely useful during formulation development and optimization, also allows setting up of product specifications and expiration dates.

The aforementioned factors were considered during formulation development and optimization of the paclitaxel emulsion in order to develop a stable and filter-sterilizable emulsion. The mean droplet diameter and 99% cumulative distribution of a typical stability lot of QW8184, lot A, are shown in Fig. 1. There were no significant changes in particle size either at 4°C or 25°C over twelve months. The actual volume-weighted particle size distribution of QW8184 incorporating 9 mg/mL of paclitaxel is shown in the insert with a mean droplet diameter of 62 nm. No precipitation or other gross changes were observed during storage. Essentially the same particle size was obtained with a small (50 mL) and a large (3 L) batch of QW8184. When the two batches were compared after one month storage at 4°C, the mean droplet/99% cumulative distribution were 67/150 nm and 64/149 nm for the small and large batches, respectively. An independently manufactured lot of QW8184, lot B, produced the following physical stability data in terms of the mean droplet diameter and 99% cumulative distribution (mean ± SD, n = 3): 63 ± 2.1 nm (initial), 62.9 ± 3.5 nm (4°C, 9 months), 59.1 ± 0.7 nm (25°C, 9 months) and 147 ± 1.9 nm (initial), 146.1 ± 2.8 nm (4°C, 9 months), 144.8 ± 1.3 nm (25 °C, 9 months), respectively.

The chemical stability of the two independently manufactured lots of QW8184, A and B, in terms of paclitaxel potency and levels of known degradants at 4°C and 25°C and at time zero, nine and twelve months is shown in Table 1. Intermediate stability data was very similar to that shown in Table 1, for clarity however, is not shown. There is no major change in the concentration of the active ingredient at least during a 12-month storage at either temperature. No significant changes in the levels of any of the degradants were observed under these storage conditions. Long-term stability is ongoing.

In Vitro Drug Release

One of the desired characteristics of a drug delivery vehicle is to provide sustained release of the incorporated drug, a characteristic quite often correlated with improved pharmacokinetics and efficacy (28,29). In particular, long-circulating emulsions

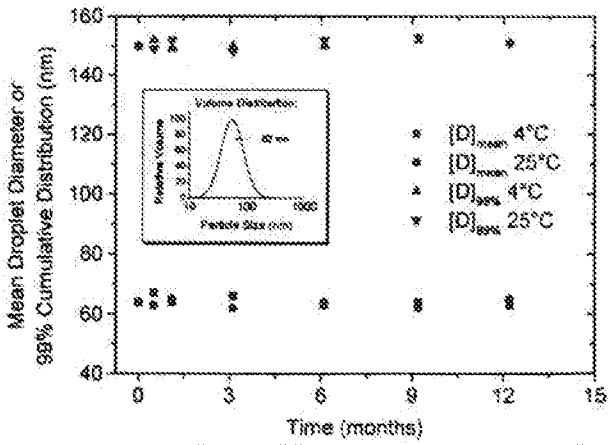

Fig. 1. Mean droplet diameter, $[D]_{mean}$, and 99% cumulative distribution, $[D]_{99\%}$, of QW8184 as a function of storage time and temperature. This particular lot of QW8184 (lot A) incorporated 9.0 mg/mL paclitaxel and was stored at 4°C or 25°C. The actual volume-weighted particle size distribution of QW8184 is shown in the insert with a mean droplet diameter of 62 nm.

Table I. QW8184 Chemical Stability: Paclitaxel Potency and Degradants

Storage time, months (Lot, temp.)	Paclitaxel potency, mg/mL (mean ± SD)[a]	Degradants (%, mean ± SD)[a]		
		7-Epi-paclitaxel	Baccatin-3	10-Deacetyl-paclitaxel
0.0 (A, 4 °C)	8.4 ± 0.6	0.61 ± 0.29	0.16 ± 0.01	0.15 ± 0.02
9.2 (A, 4 °C)	9.2 ± 0.1	0.36 ± 0.02	0.17 ± 0.00	0.18 ± 0.01
12.2 (A, 4 °C)	8.8 ± 0.3	0.30 ± 0.04	0.21 ± 0.04	0.20 ± 0.03
9.2 (A, 25 °C)	9.0 ± 0.7	0.40 ± 0.02	0.18 ± 0.01	0.18 ± 0.01
12.2 (A, 25 °C)	8.3 ± 0.8	0.35 ± 0.07	0.21 ± 0.04	0.21 ± 0.04
0.0 (B, 4 °C)	9.1 ± 0.0	0.50 ± 0.07	0.07 ± 0.01	0.11 ± 0.01
9.2 (B, 4 °C)	9.4 ± 0.0	0.43 ± 0.03	0.08 ± 0.01	0.13 ± 0.02
9.2 (B, 25 °C)	9.2 ± 0.1	0.41 ± 0.04	0.08 ± 0.01	0.13 ± 0.01

[a] n = 10 (lot A); n = 3 (lot B).

of paclitaxel can improve the delivery of the drug to cancer sites in the body. The release kinetics of paclitaxel from QW8184, both in the absence and presence of human serum albumin (HSA) at a drug to albumin molar ratio of 1000:1 and from the commercial formulation Taxol® are shown in Fig. 2. The concentration of paclitaxel in Taxol® and QW8184 was 6.0 and 8.8 mg/mL, respectively. The release of paclitaxel from the emulsion was slow both in the absence and presence of serum albumin with less than 5% drug being released over 24 hours, whereas about 12% was released from the commercial formulation.

Acute Single Dose Toxicity

Single dose toxicity studies of paclitaxel emulsions in healthy mice were carried out and representative data with QW8184 is shown in Fig. 3. The paclitaxel emulsion was well tolerated and the maximum tolerated dose (MTD) was determined to be approximately 70 mg/kg for QW8184 as compared to approximately 20 mg/kg for Taxol®. The marketed drug product Taxol® is primarily supplied in 5 mL vials at a drug concentration of 6 mg/mL and prior to administration it requires dilution with commonly used i.v. fluids to a final concentration of 0.3 to 1.2 mg/mL that corresponds to a 20-fold and 5-fold dilution, respectively. When Taxol® was administered i.v. at 24 mg/kg, either as a bolus dose or 2 min infusion, after a 10-fold dilution with saline, severe prostration and catatonia were observed. When Taxol® was first diluted 1:4 with saline and then administered i.v. at 24 mg/kg as a 2 or 5 min infusion, 100% mortality was observed as compared to 50% mortality when the drug was administered as i.v. bolus. Weight loss was less than 15% even in the group receiving the highest dose of QW8184 and the animals recovered or gained weight over a period of 10 days post injection. Vehicle toxicity was also evaluated. Animals receiving drug-free emulsion at 7 or 8 mL/kg (the equivalent paclitaxel dose of 63–72 mg/kg at drug loading of 9.0 mg/mL) grew rapidly and gained slightly more weight than animals receiving saline. This may be attributed to the vitamin and calorie content of the formulation. The clinical dose of paclitaxel is 175 mg/m^2 (300 mg total per person) or 4.3 mg/kg for a 70 kg average human body weight (1,23). The equivalent dose/volume of QW8184 is about 0.5 mL/kg at a drug loading of 9.0 mg/mL which is about an order of magnitude below the dose where the emulsion vehicle may show signs of toxicity. The high drug loading in QW8184

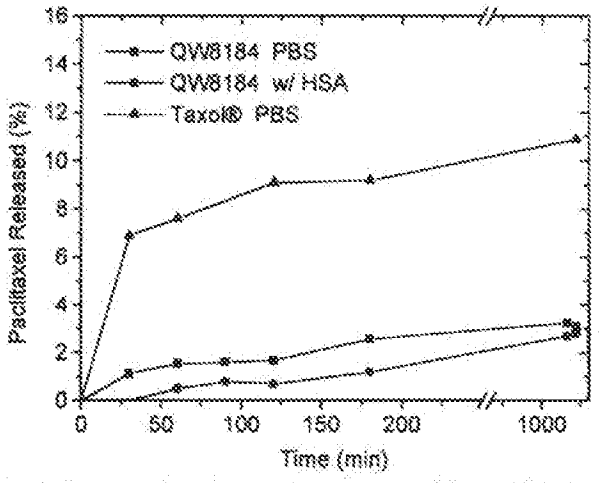

Fig. 2. Percent paclitaxel released as a function of time at 37°C. Drug release from the lipid emulsion, QW8184, in the presence and absence of HSA (human serum albumin) and the commercial formulation Taxol® was monitored by membrane dialysis as described in Methods using PBS (phosphate-buffered saline) as a sink solution at pH 7.0. The drug to albumin molar ratio was 1000:1.

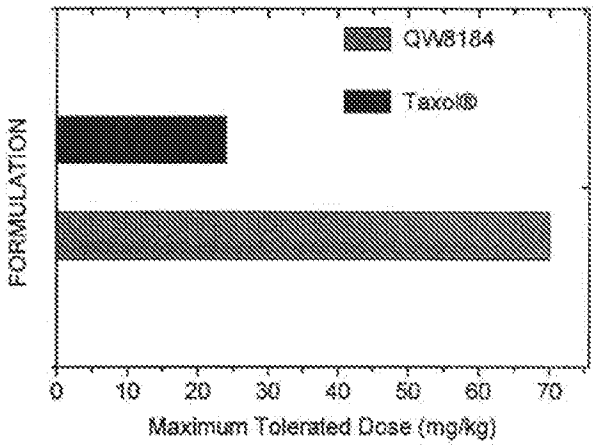

Fig. 3. Maximum tolerated dose of paclitaxel in mice. The determination of the MTD was based on body weight loss (>15% is considered toxic) and mortality (number of deaths per group of 6 animals).

without prior dilution enables one to administer much lower volumes, therefore reducing or potentially eliminating issues of vehicle toxicity.

Efficacy Against B16 Melanoma

B16 Melanoma is a fast growing solid murine tumor that has been commonly used in early screening of different anticancer agents (23,24). Figures 4, A and B present tumor growth regression and percent survival, respectively, for the dosing regimen of q3d × 5. QW8184 was evaluated at different doses and compared to the reference Taxol® formulation at its MTD (20 mg/kg). The corresponding data for the dosing schedule of q4d × 5 are shown in Figure 5, A and B, respectively. QW8184 illustrated a definitive dose response in both schedules. The q3d × 5 schedule, however, seems to be more effective. Administration of QW8184 at dosages of 20 mg/kg (63 mg/m^2) and 40 mg/kg (125 mg/m^2) on a schedule of q3d × 5 resulted in mean increases in survival times of 65% and 94%, respectively, as compared to control group (Table 2).

Log-cell kill values of 1.8 and 3.0 were observed with QW8184 at dosages of 20 and 40 mg/kg, respectively (Table 2), as compared to a value of 0.5 obtained with Taxol® at 20 mg/kg (q3d × 5). In addition, there was a significant reduction in tumor growth in these animals as depicted in Fig. 4A. A significant reduction in tumor growth was also observed in animals administered QW8184 on a schedule of q4d × 5 as illustrated in Fig. 5A. A dosage of 60 mg/kg with QW8184 was toxic in the q3d × 5 regimen with only 1 survivor at the end of the dosing schedule (Fig. 4B). In the q4d × 5 dosing schedule, 70 mg/kg of QW8184 was toxic with only 2 survivors at the end of the dosing schedule (Fig. 5B). Statistical analysis (t-test) of the survival time between groups was performed. The results indicate that Taxol® was not statistically different from the saline control at the q3d × 5 ($p = 0.25$) or q4d × 5 ($p = 0.09$) schedule. QW8184 resulted in a statistically significant increase in survival time in the q3d × 5 groups at dosages of 20 mg/kg ($p = 0.005$) and 40 mg/kg ($p < 0.005$) as compared

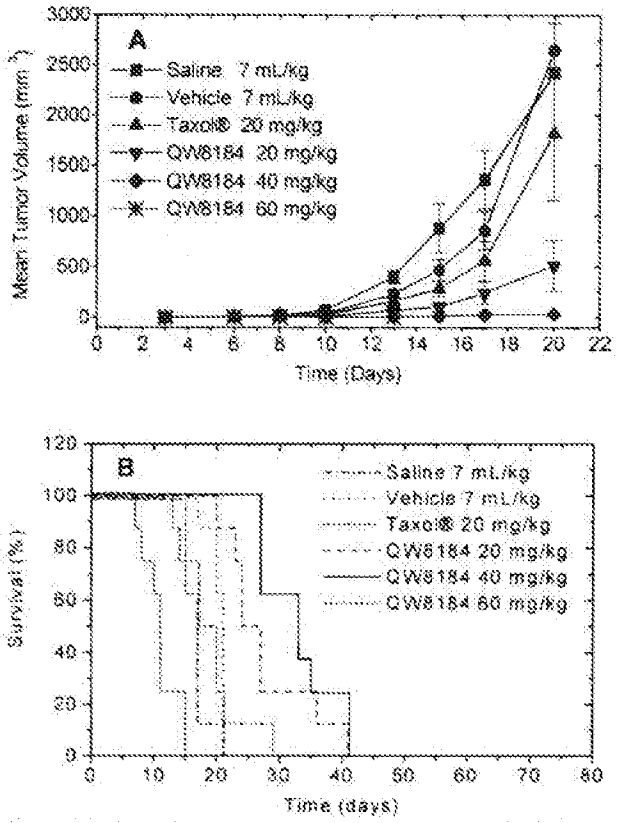

Fig. 4. A) B16 melanoma mean tumor regression to QW8184 and Taxol® as a function of time on a q3d × 5 schedule. Error bars represent SEM ($n = 8$). B) Percent B16 melanoma survival in response to QW8184 and Taxol® as a function of time on a q3d × 5 schedule.

Table 2. Antitumor Activity of QW8184 vs Taxol® in the B16 Melanoma Model

Test article	Dose mg/kg n = 8	Schedule days	Median tumor weight on day 3	15	20	23	34	Survival (mean ± SD) days	% Mortality day 20	% T/C[a] day 20	% TGI[b] day 20	T-C[c] days	Log cell kill[d]
Saline	Control	q3d x 5	0	699	2422	----	----	17 ± 2	87.5	---	---	---	---
Vehicle	Control	q3d x 5	0	388	2358	----	----	20 ± 1	0[e]	93	3	3	---
Taxol®	20	q3d x 5	0	306	1871	----	----	19 ± 6	50	77	23	3	0.5
QW8184	20	q3d x 5	0	50	270	903	----	28 ± 7	0	11	89	10	1.8
QW8184	40	q3d x 5	0	7	6	139	1340	33 ± 5	0[f]	0	100	17	3.0
QW8184	60	q3d x 5	0	----	----	----	----	11 ± 3	100	---	---	---	---
Vehicle	Control	q4d x 5	0	400	2658	----	----	16 ± 6	62.5	100	0	0	0
Taxol®	20	q4d x 5	0	184	1679	----	----	12 ± 7	75	69	31	3	0.5
QW8184	20	q4d x 5	0	223	855	1079	----	20 ± 3	50	35	65	3	0.5
QW8184	30	q4d x 5	0	49	246	407	----	31 ± 4	0	10	90	7	1.2
QW8184	70	q4d x 5	0	2	2	15	1577	17 ± 16	62.5	0	100	17	3.0

[a] % T/C = (Median Tumor Wt of treated / Median Tumor Wt of control) × 100.
[b] %TGI = 100 − (%T/C).
[c] T-C = Tumor Growth Delay Value (median time for the treatment group (T) and control (C) to reach a predetermined size (> 750 mg).
[d] Log Cell Kill = (T-C value)/(3.32 × tumor doubling time).
[e] All animals were sacrificed due to excessive tumor size.
[f] All animals but three survived to day 27 when they were sacrificed due to tumor size (>10% of body weight).

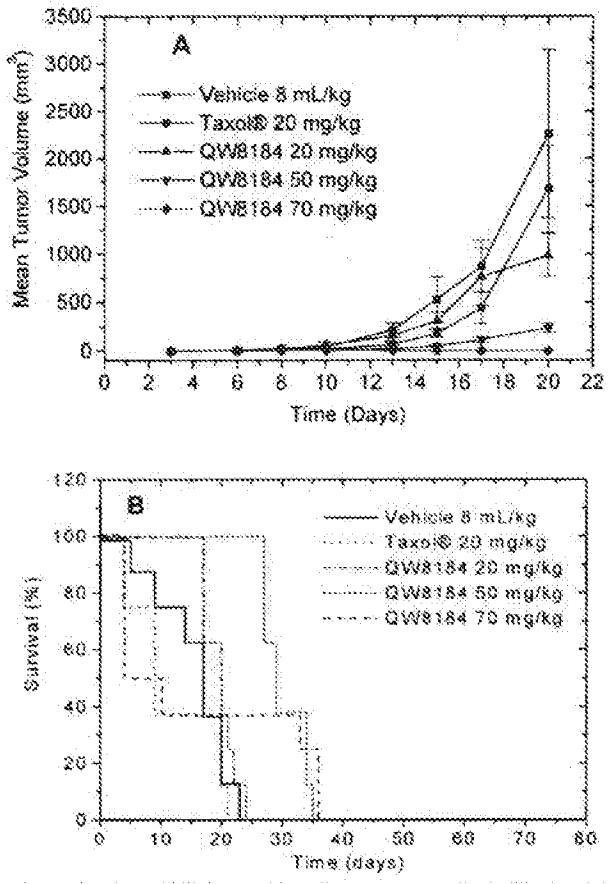

Fig. 5. A) B16 melanoma mean tumor regression to QW8184 and Taxol® as a function of time on a q4d × 5 schedule. Error bars represent SEM (n = 8). B) Percent B16 melanoma survival in response to QW8184 and Taxol® as a function of time on a q4d × 5 schedule.

to the saline control. The animals administered QW8184 at q4d × 5 resulted in a statistically significant increase in survival time at dosages of 20 mg/kg ($p = 0.05$) and 50 mg/kg ($p < 0.005$) as compared to the saline control.

Table 2 summarizes the overall results of the efficacy study including mean survival times, median tumor weights and log cell kills for each group and treatment schedule. By all end points of efficacy (24), QW8184 exhibited superior antitumor activity in mice at doses that included or well exceeded the MTD of Taxol® but which were well tolerated. In our laboratory, the MTD for Taxol® was approximately 20 mg/kg and it is supported by other literature reports. Bissery et. al. (30) reported that the highest dose of Taxol® that could be administered to mice without causing death or undue toxicity was 21.7 mg/kg/injection. Furthermore, recent reports on the MTD of Taxol® upon i.v. administration in mice indicate that it is 20 mg/kg on a q1d × 3 (31) or 25 mg/kg on a q1d × 5 (32). From Table 2, it is evident that Taxol® did result in a slight reduction in tumor growth at both the 15 and 20 days post tumor implant as compared to the controls. It should also be noted that Taxol® has been shown to be minimally effective in the B16 melanoma tumor model (33). In addition, drug related mortality is evident at day 20 in the Taxol® group thus providing further evidence that doses higher than 20 mg/kg could not have been tolerated. Therefore, the observed minimal therapeutic effect from Taxol® in the B16 melanoma model is not believed to be due to sub-optimal dosing.

The effects observed in the present study have not been reported with previous injectable emulsions of paclitaxel (7–10). The improved efficacy of QW8184 may be related to its preferential uptake by tumor cells as a result of its physicochemical characteristics, particularly lipid composition and particle size. The *in vitro* drug release data suggest that emulsion droplets may serve as long-circulating drug reservoirs, thus improving the delivery of paclitaxel to tumor sites, due to increased cellular/droplet interactions (34). To penetrate tumors in tissue, particles must be small enough to pass through endothelial fenestrae, i.e. < 70 nm. This mechanism is consistent with literature reports on long-circulating emulsions and liposomes (29,34) where lipid composition, particularly the inclusion of pegylated surfactants and small droplet size, direct lipophilic drugs away from RES in the liver and spleen to other targeting tissues, such as inflammatory tissues.

In addition to the studies reported with drug emulsions, toxicity and antitumor activity was reported with other lipid-based carriers of paclitaxel, such as liposomes and mixed micelles. The most extensive work with liposomal formulations of paclitaxel has been reported by Straubinger *et al*; (3,12,13). They developed over 300 liposome formulations of various lipid compositions and evaluated them for stability and antitumor activity. Both *in vitro* and *in vivo* activity using Colon-26, a Taxol-resistant murine tumor, were demonstrated (12). Paclitaxel liposomes, however, were both unstable and toxic at high drug loading.

Paclitaxel was solubilized in mixed micelles formed by a mixture of bile salts and phospholipids followed by a spontaneous transformation into drug-loaded liposomes, thus avoiding drug precipitation (14). These formulations, incorporating less than 1.0 mg of paclitaxel per mL, produced significant antitumor activity *in vitro* and appeared to be less toxic than the Cremophor EL vehicle (14). It is anticipated however, that at high drug loading these formulations can be both unstable and toxic with limited clinical use.

Emulsion and liposomal formulations of paclitaxel are more biocompatible and thus less toxic and at least as efficacious as the marketed Taxol®. Emulsions have higher drug solubilization capacity than liposomes and are easier to process and manufacture in a sterile form. The injectable paclitaxel emulsion QW8184 described in the present studies exhibits these advantages. Due to the high paclitaxel loading (8–10 mg/mL), QW8184 can be administered clinically undiluted at high dose without drug precipitation. As a result of its small droplet size (<200 nm for both the mean droplet and cumulative distribution), QW8184 can be filter-sterilized, exhibits improved shelf-life, efficacy and targeting to solid tumors. Furthermore, QW8184 can result in better quality of life, since it can be administered to patients as a bolus dose and has low toxicity. Emulsion formulations offer an appealing alternative for the bolus administration of paclitaxel and other poorly soluble drugs due to their effectiveness for drug solubilization, improved efficacy and patient quality of life arising from reduced side effects. A real therapeutic gain with a particular new formulation of paclitaxel, including QW8184, can be realized through its

progression to the clinic. As new and safer drug delivery methods of paclitaxel emerge, it is likely that the clinical use of the drug will expand.

CONCLUSIONS

QW8184, a stable, injectable and filter sterilizable o/w emulsion of paclitaxel has been developed at high drug loading (8–10 mg/mL) with a mean droplet diameter and 99% cumulative particle size distribution of < 0.2 μm. Compared to the *in vitro* drug release from Taxol®, drug release from QW8184 at 37°C is slow both in the presence and absence of human serum albumin with less than 5% drug being released within 24 hours. In the B16 melanoma tumor model in mice the drug emulsion was better tolerated and was more efficacious than Taxol®. The composition and dependent physicochemical characteristics of the emulsion may be related to reduced toxicity and improved efficacy.

ACKNOWLEDGMENTS

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Schupsky, S. Ali, A. S. Janoff, and Mayhew, E. Therapeutic efficacy of a novel polyethyleneglycol (PEG)-lipid formulation of 2′ [alpha]-bromo-hexedecanoyl paclitaxel (BrC16HT) against human tumor xenografts in SCID mice. *Proc. Amer. Assoc. Cancer Res.* **40**:#3849 (1999).
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m-Anisidine. 3-methoxybenzenamine, 3-methoxyaniline, 3-aminoanisole. Pale yellow, oily liquid. Remains fluid even at −10°. bp 251°; bp_5 81-86°. Sparingly sol in water; sol in alc, acids.

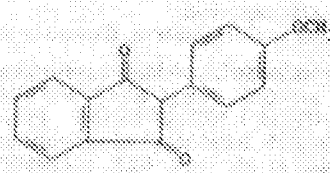
o-Anisidine. 2-methoxybenzenamine. Yellowish liquid; becomes brownish on exposure to air. Volatile with steam. bp 225°. mp +5°. d_4^{25} 1.098. Practically insol in water. Miscible with alc, ether. *Keep well closed and protected from light.*

p-Anisidine. 4-methoxybenzenamine. Crystals, mp 57°, bp 246°. Sparingly sol in water; freely sol in methanol, ethanol.

Note: o-Anisidine and its hydrochlorides may reasonably be anticipated to be carcinogens. *Fourth Annual Report on Carcinogens* (NTP 85-002, 1985) p 34.

USE: In the manuf of azo dyes.

698. Anisindione. *2-(4-Methoxyphenyl)-1H-indene-1,3(2H)-dione;* 2-*p*-anisyl-1,3-indandione; 2-(*p*-methoxyphenyl)-1,3-indandione; SPE 2782; Miradine; Umidone. $C_{16}H_{12}O_3$; mol wt 252.26. C 76.18%, H 4.80%, O 19.03%. Prepn: Kowitch, *J. Am. Chem. Soc.* **58**, 1331 (1936); Horeau, Jacques, *Bull. Soc. Chim. France* **1948**, 53; Sperber, U.S. pat. 3,009,388 (1959 to Schering).



Pale yellow crystals from acetic acid or ethanol, mp 156-157°.

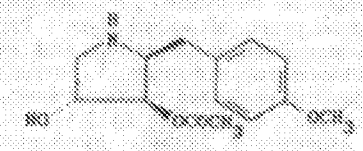
THERAP CAT: Anticoagulant.

699. Anisole. *Methoxybenzene.* C_7H_8O; mol wt 108.13. C 77.75%, H 7.46%, O 14.80%. $C_6H_5OCH_3$. Prepn from phenol and dimethyl sulfate: Ullmann, *Ann.* **327**, 114 (1903); Graebe, *Ann.* **340**, 208 (1905); G. S. Hiers, F. D. Hager, *Org. Syn.* coll. vol. I, 58 (2nd ed., 1941); from bromobenzene: Agfa, *Ger. pat.* 411,852; *Chem. Zentr.* **1925**, I, 2411; *Frdl.* **15**, 193; by passing methyl chloride into a suspension of sodium phenolate in liquid ammonia: White *et al., J. Am. Chem. Soc.* **48**, 865 (1926); from phenol, methyl iodide and potassium carbonate in dimethylformamide: Brieger *et al., J. Chem. Eng. Data* **13**, 581 (1968). Forms oils or resins by condensation with formaldehyde. *Ger. pats.* 403,268, 406,152; *Chem. Zentr.* **1925**, I, 307, 1816; *Frdl.* **14**, 826, 827. Absorption spectrum: Scheibe, *Ber.* **59**, 2625 (1926). Soly in glycerol, see McEwen, *J. Chem. Soc.* **123**, 2285 (1923). Toxicity studies: J. M. Taylor *et al. Toxicol. Appl. Pharmacol.* **6**, 378 (1964).

Liquid. Agreeable aromatic odor. d_4^{20} 0.9956; d_4^{25} 0.9901; mp −37.5°; bp_{760} 155.5°; bp_{400} 91.0°; bp_{40} 70.7°; bp_{20} 58.8°; bp_{10} 42.2°; bp_5 30.0°; $bp_{1.5}$ 5.6°; n_D^{20} 1.51791. Sol in alcohol and ether; insol in water. LD_{50} orally in rats: 3700 mg/kg (Taylor).

USE: In perfumery, in organic syntheses.

700. Anisomycin. *1,4,5-Trideoxy-1,4-imino-5-(4-methoxyphenyl)-D-xylo-pentitol 3-acetate; [2R-(2α,3α,4β)]-2-[(4-methoxyphenyl)methyl]-3,4-pyrrolidinediol 3-acetate; 2-p*-methoxyphenylmethyl-3-acetoxy-4-hydroxypyrrolidine; Flagecidin. $C_{14}H_{19}NO_4$; mol wt 265.30. C 63.38%, H 7.22%, N 5.28%, O 24.12%. Protein synthesis inhibiting antibiotic isolated from *Streptomyces griseolus* and *S. roseochromogenes*: Sobin, Tanner, *Jr., J. Am. Chem. Soc.* **76**, 4053 (1954); Tanner *et al.*, U.S. pat. 2,681,618 (1954 to Pfizer). Activity: J. E. Lynch *et al., Antibiot. & Chemother.* **4**, 844, 849 (1954). Structure and stereochemistry: Beereboom *et al., J. Org. Chem.* **30**, 2334 (1965); Schaefer, Wheatley, *ibid.* **33**, 166 (1968); Butler, *ibid.* 2136. Biosynthesis: Butler, *ibid.* **31**, 317 (1966). Total synthesis: Ogata, Ohki, *Chem. Pharm. Bull.* **16**, 2088 (1968); *ibid.* **17**, 1405 (1969); Fehrer, Schenk-er, *Helv. Chim. Acta* **53**, 758 (1970). Chiral synthesis: J. P. H. Verheyden *et al., Pure Appl. Chem.* **50**, 1363 (1978). Stereospecific total synthesis: D. F. Schumacher, S. S. Hall, *J. Am. Chem. Soc.* **104**, 6076 (1982). Mechanism of action: A. Jiménez, D. Vázquez in *Antibiotics* vol. 3(pt. 2), F. E. Hahn, Ed. (Springer-Verlag, New York, 1979) pp 1-18. Solubility and stability data: *Antibiot. Ann.* **1958-59**, pp 808-810. Prepn of deacetylanisomycin from anisomycin: Nickell *et al.*, U.S. pat. 2,915,444 (1960 to Pfizer).



Long needles from ethyl acetate or water, mp 140-141° $[\alpha]_D^{25}$ −30° (methanol). uv max: 224, 277, 283 nm (ε 10800, 1800, 1600). Base is moderately sol in water, sol in lower alcohols, esters, ketones, chloroform; slightly sol in benzene, toluene and hexane. Aq solns are stable over a wide pH range at room temp.

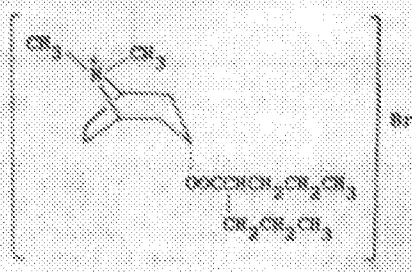
Hydrochloride. $C_{14}H_{19}ClNO_4$; crystals from ethyl acetate + ethanol, mp 187-188°. Very sol in water.

Deacetylanisomycin. $C_{12}H_{17}NO_3$; mp 176-179°. $[\alpha]_D^{25}$ −20.0° (methanol). pK 9.2.

USE: Anisomycin and deacetylanisomycin in the eradication of bean mildew; to inhibit other pathogenic fungi in plants.

THERAP CAT: Antiprotozoal (Trichomonas).

701. Anisotropine Methylbromide. *endo-8,8-Dimethyl-3-[(1-oxo-2-propylpentyl)oxy]-8-azoniabicyclo[3.2.1]octane bromide; 3α-hydroxy-8-methyl-1αH,5αH-tropanium bromide 2-propylvalerate; 8-methyltropinium bromide 2-propylvalerate; 8-methyl-3-(2-propylpentanoyloxy)tropanium bromide; octatropine methylbromide;* Lytispasm; Valpas. $C_{18}H_{32}BrNO_2$; mol wt 374.37. C 57.75%, H 8.62%, Br 21.34%, N 3.74%, O 8.55%. Prepn: Werner, Gordon, U.S. pat. 2,963,498 (1960 to Endo Labs.). Metabolism: Shinde *et al., Chem. Pharm. Bull.* **19**, 513 (1971).

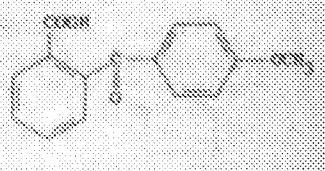


Crystals from acetone, mp 159°.

Methyl chloride. $C_{19}H_{34}ClNO_2$; crystals from acetone, mp 209°.

THERAP CAT: Anticholinergic.

702. *o*-(*p*-Anisoyl)benzoic Acid. *2-(4-Methoxybenzoyl)benzoic acid;* S 23746. $C_{15}H_{12}O_4$; mol wt 256.25. C 70.30%, H 4.72%, O 24.98%. Prepn from phthalic anhydride and anisole: Meyer, Turnau, *Monatsh.* **30**, 488 (1909). Alternate route: Arcus, Marks, *J. Chem. Soc.* 1956, 1527.



Leaflets from water. Short crystals from alcohol or toluene, mp 146°. Very sparingly sol in water. Freely sol in alc, ether, toluene, chloroform, glacial acetic acid.

Sodium salt. $C_{15}H_{11}NaO_4$; needles. Freely sol in water. Sol in alcohol.

USE: The sodium salt has been proposed as a sweetening agent. Rated approximately 150 times as sweet as cane sugar. Bitter taste if used in amount exceeding 0.2 g/liter. Review: Mühler, *Z. Lebensm.-Untersuchung u. -Forschung* **90**, 431 (1950); *C.A.* **44**, 8558d (1950).

703. *p*-Anisoyl Chloride. *4-Methoxybenzoyl chloride.* $C_8H_7ClO_2$; mol wt 170.59. C 56.32%, H 4.14%, Cl 20.78%, O 18.76%. $CH_3OC_6H_4COCl$. Prepd from *p*-anisic acid and

Colorless needles from ethanol, mp 235–237° (dec). LD_{50} in male, female mice, male, female rats (mg/kg): 5450, 5250, 3580, 4750 orally; 268, 233, 273, 236 i.v.; >10000, >10000, 7070, 9000 s.c. (Oltrez).

THERAP CAT: Antibacterial.

6689. Oil of Amber, Rectified. Obtained by the destructive distillation of amber and purified by redistillation. Consists of a mixture of terpenes with resinous, oxygen-containing substances.

Pale yellow to yellowish-brown, volatile oil; penetrating odor; burning acrid taste. d 0.850–0.920; α_D^{20} +22° to +26°; fixed in water. Sol in about 10 vols alcohol; freely sol in chloroform, ether, carbon disulfide, oils.

6690. Oil of Angelica. Volatile oil from root of *Angelica archangelica* L. (*A. officinalis* Moench.). *Umbelliferae*. Constit: Phellandrene, valeric acid.

Yellow liquid. d_{25}^{25} 0.857–0.915; α_D^{20} +16° to +32°; n_D^{20} 1.4500. Almost insol in water; sol in 6 vols 90% alcohol. *Keep well closed, cool, and protected from light.*

USE: In muscat liqueurs.

6691. Oil of Anise. Volatile oil from dried ripe fruit of *Pimpinella anisum* L., *Umbelliferae*, or of *Illicium verum* Hook. fil., *Magnoliaceae* (Chinese star anise). Constit: 80–90% anethole, methylchavicol, anisaldehyde.

Colorless or pale yellow, refractive liquid. d_{25}^{25} 0.978–0.988. Solidifies below 15°; α_D +3° to −2°. n_D^{20} 1.553–1.560. Slightly sol in water; sol in about 3 vols alcohol, freely sol in chloroform, ether. *Keep cool in well-closed and well-filled containers, protected from light.*

USE: In manuf of liqueurs; flavor for candies, cookies, dentifrices. Pharmaceutic aid (flavor).

THERAP CAT: Carminative, expectorant.

THERAP CAT (vet): Has been used as a carminative.

6692. Oil Anise, Japanese. Volatile oil from fruit of *Illicium anisatum* L., *Magnoliaceae* (Japanese star anise). Constit: Chiefly anethole; also safrol, eugenol.

Colorless to slightly yellow liquid. d about 1.006. Solidifies at −10° to −15°. α_D^{20} about −8°.

THERAP CAT: Carminative; expectorant.

6693. Oil of Asarum. Oil of Canada snakeroot. Volatile oil from rhizome of *Asarum canadense* L., *Aristolochiaceae*. Constit: Terpene (pinene), methyleugenol, borneol, linalool, geraniol.

Yellowish-brown liq; aromatic odor and taste. d_{25}^{25} 0.93–0.96; α_D^{20} −1.4 to −3.5°. Practically insol in water; sol in 2 vols 70% alc. *Keep well closed, cool and protected from light.*

6694. Oil of Balm. Oil of melissa balm; oil of lemon balm. Volatile oil from leaves and tops of *Melissa officinalis* L., *Labiatae*. Chiefly citral. Composition studies: Helendahl, *Arch. Pharm.*, **303**, 343 (1970).

Yellow to yellowish green liquid. d_{25}^{25} 0.89–0.925. Practically insol in water; sol in alcohol. *Keep well closed, cool, and protected from light.*

6695. Oil of Basil. Volatile oil from leaves of *Ocimum basilicum* L., *Labiatae* (sweet basil). Constit: Methylchavicol, eugenol, linalool, estragol.

Yellowish to greenish liquid; aromatic odor. d_{25}^{25} 0.905–0.930; α_D^{20} −8° to −22°. Almost insol in water; sol in 2 vols 80% alc; miscible with ether, chloroform. *Keep well closed, cool and protected from light.*

6696. Oil of Bay. Oil of Myrcia. Volatile oil distilled from leaves of *Pimenta* (*Myrcia*) *acris* Kostel., *Myrtaceae*. Constit: 40–55% eugenol; myrcene, chavicol, methyleugenol, methylchavicol, citral; hydrocarbons; total phenols, 50–65% by volume.

Yellow to brownish-yellow liq; pleasant odor; sharp, spicy taste; becomes brown on exposure to air. d_{25}^{25} 0.962–0.968; α_D^{20} −7° to −9°. n_D^{20} 1.520–1.530. Insoluble in water. Very sol in alcohol, carbon disulfide, glacial acetic acid.

USE: Pharmaceutic aid (aromatic). Manuf bay rum.

6697. Oil Bergamot. Volatile oil expressed from rind of fresh fruit of *Citrus aurantium* L. var. *bergamia* Wight & Arn., *Rutaceae*. Constit: 36–45% linalyl acetate, about 6% linalool; d-limonene, dipentene, bergaptene.

Yellowish green liquid; agreeable odor. d_{25}^{25} 0.875–0.890; α_D^{20} +8° to +24°. n_D^{20} 1.464–1.467. Acid no. 1–6. Almost insol in water; sol in 0.5 vol 95% alcohol, 3 vols 80% alcohol. *Keep well closed in a cool place, protected from light.*

USE: For masking many disagreeable odors, such as sulfonal, naphthalene, etc. In perfumery, hair oils, pomades.

6698. Oil of Bitter Almond. Volatile oil from dried ripe kernels of bitter almonds or from other kernels containing amygdalin, such as apricots, cherries, plums, and injured peaches. Obtained by macerating with water, then by distilling. Constit: Not less than 95% benzaldehyde; HCN and phenoxyacetonitrile.

Colorless to yellow, very refractive liq; characteristic odor and taste of benzaldehyde. Very poisonous! d_{25}^{25} 1.038–1.060; n_D^{20} 1.5428–1.5439. Slightly sol in water; miscible with alcohol, ether, oils. *Keep cool and protected from light.*

Human Toxicity. Hydrogen cyanide (q.v.) component responsible for highly toxic properties.

USE: Only the oil *free from HCN* may be used for food and foods.

THERAP CAT: Formerly as topical antipruritic.

6699. Oil of Bitter Orange. Volatile oil expressed from fresh peel of *Citrus aurantium* L., *Rutaceae*. Constit: 93–96% d-limonene; citral, decyl aldehyde, methyl anthranilate, linalool, terpineol.

Pale yellow liquid; bitter taste. d_{25}^{25} 0.842–0.848; α_D^{20} +90°. Very slightly sol in water; miscible with alcohol; sol in 4 vols alcohol, in 1 vol glacial acetic acid. *Keep well closed, cool, and protected from light.*

USE: As flavoring in perfumery.

6700. Oil of Cajeput. Cajeput oil; cajeput oil. Volatile oil from fresh leaves and twigs of several varieties of *Melaleuca leucadendron* L., and other species of *Melaleuca*, *Myrtaceae*. Constit: 50–60% eucalyptol (cineol); l-pinene, camphene, butyric, benzoic and other aldehydes. Toxicity: P. M. Jenner *et al.*, *Food Cosmet. Toxicol.* **2**, 1 (1964).

Colorless or yellowish liquid; agreeable camphor odor and bitter aromatic taste. d 0.912–0.925; α_D^{20} −6 to +4°; n_D^{20} 1.4660–1.4710. Very slightly sol in water; sol in 1 vol alcohol. Miscible with alcohol, chloroform, ether, carbon disulfide. *Keep well closed, cool, and protected from light.* LD_{50} orally in rats: 3870 mg/kg (Jenner).

THERAP CAT: Expectorant, counterirritant, sedative.

THERAP CAT (vet): Rubefacient, topical antipyretic.

6701. Oil of Calamus. Oil of sweet flag. Volatile oil from rhizome of *Acorus calamus* L., *Araceae* grown in N. America, Europe and Asia. Constit: *cf.* Jamrog, Indian Perfy: d-asarone 75.8%; calamene 3.84%; calamol 3.7%; asarone 1.32%; camphene 0.92%; β-pinene 0.56%; acetaldehyde 0.2%. Vashist, Handa, *Soap Perfum. Cosmet.* **37**, 1 (1964).

Yellow to yellowish-brown viscid liquid; aromatic odor; bitter taste. d_{25}^{25} 0.960–0.970. α_D^{20} +9° to +31°. n_D^{20} 1.512–1.515. Sapon no: 16–30. Very slightly sol in water; miscible with alcohol. LD_{50} orally in rats (Jenner's variety): mg/kg: 3. M. Taylor *et al.*, *Toxicol Appl. Pharmacol.* **8**, 97 (1967). *Keep well closed, cool and protected from light.*

USE: In perfumery. Formerly as minor ingredient of flavors such as vermouth and flavored wines.

6702. Oil of Camphor, Rectified. Formosa oil of camphor; Japanese oil of camphor; white oil of camphor; yellow oil of camphor. Volatile oil from *Cinnamomum camphora* Nees & Eberm., *Lauraceae*. Constit: Safrol, acetaldehyde, camphor, terpineol, eugenol, cineol, d-pinene, phellandrene, dipentene, sabinene.

Colorless or yellowish liquid; camphor odor. d_{25}^{25} 0.870–0.900; α_D^{20} +9° to +38°. n_D^{20} 1.465–1.470. Insol in water; sol in chloroform, ether, oils, in about 3 vols alcohol. *Keep well closed, cool, and protected from light.*

USE: As solvent in paint and lacquer industry; in polishing of soaps and detergents; in technical odor masking.

THERAP CAT: Rubefacient.

6703. Oil of Caraway. Volatile oil distilled from ripe fruit of *Carum carvi* L., *Umbelliferae*. Constit: 50–60% carvone (by vol); d-limonene. Composition studies: